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REGULATION OF TRYPANOSOMA BRUCEI HEXOKINASE 1 AND 2 ON MULTIPLE LEVELS: TRANSCRIPT ABUNDANCE, PROTEIN EXPRESSION AND ENZYME ACTIVITY

Heidi Dodson
Clemson University, heidid@clemson.edu

REGULATION OF TRYPANOSOMA BRUCEI HEXOKINASE 1 AND 2 ON MULTIPLE LEVELS: TRANSCRIPT ABUNDANCE, PROTEIN EXPRESSION AND ENZYME ACTIVITY

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Heidi Cornelia Dodson
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Accepted by:
Dr. James C. Morris, Committee Chair
Dr. Kimberly S. Paul
Dr. Michael G. Sehorn
Dr. Kerry S. Smith

ABSTRACT

Trypanosoma brucei, a unicellular eukaryotic parasite, is the causative agent of African sleeping sickness in sub-Saharan Africa. The parasite encounters two main environments as it progresses through its life cycle: the tsetse fly and the mammalian bloodstream. Nutrient availability is distinct in the two environments, requiring the parasite to utilize diverse metabolic pathways to efficiently produce ATP for survival. Bloodstream form parasites (BSF), residing in a glucose rich environment, rely solely on glycolysis for energy, while procyclic form (PF) parasites metabolize readily available proline and threonine in addition to glucose.

T. brucei expresses two hexokinases, the first enzyme in the glycolytic pathway, that are 98.5% identical at the nucleotide level and 98% similar at the amino acid level. These two enzymes, TbHK1 and TbHK2, are differentially expressed in both BSF and PF parasites. Here, I identify a means of regulation of *TbHK1* gene expression and a novel mechanism for regulating TbHK1 enzyme activity. Lastly, I have characterized, the bioflavonoid quercetin, a small molecule regulator TbHK1.

Mechanisms involved in regulating *TbHK1* and *TbHK2* gene expression have not been extensively studied. I have found that *TbHK1* uses differential polyadenylation to regulate gene expression in variable environmental conditions. A recent study by Siegel et al. (2010), revealed that *TbHK1* contains seven predominant polyadenylation sites, whereas, *TbHK2* contains only one. Using a reporter gene system to assess transcript level and protein expression, I have determined that these seven different 3'UTR lengths

result in differential steady state transcript abundance and expression level, dependent on nutrient availability.

Further, I have identified a novel means of regulation of TbHK1 enzyme activity. Enzymatic studies of recombinant TbHK1 reveal that TbHK is inactivated under acidic conditions. However, the addition of glycerol-3-phosphate to the reaction at acidic pH maintained *T. brucei* hexokinase activity. I propose this regulation may play an important role in the biology of the parasite during differentiation and subsequent acidification of glycosomes, the peroxisomal like organelle that houses glycolytic enzymes.

Lastly, the TbHKs were identified as possible drug targets because they are essential to the BSF parasite and only ~30% similar to human glucokinase, the human equivalent of TbHK1. We have found that quercetin, a known trypanocide, is a potent inhibitor of TbHK1. Further, taking advantage of the fluorescent nature of the compound, I found that the compound localizes to the same subcellular compartment that houses TbHK1 and interacts with the protein near the active site as a mixed inhibitor.

DEDICATION

For my family:

Daddy and Mama~

Thank you for teaching me the value of kindness and hard work.

Jason, Alicia, Abby & Jake~

Thank you for your continuous love and support.

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CHAPTER ONE

LITERATURE REVIEW

Trypanosoma brucei, a single-celled eukaryotic parasite, is the causative agent of African sleeping sickness, affecting 50-70,000 people in 36 countries of sub-Saharan Africa (Brun, 2010). There are three subspecies of *T. brucei*: *T. brucei brucei, T. brucei rhodesiense,* and *T. brucei gambiense*, with the latter two subspecies being infective to humans. *T. b. gambiense* is endemic to west and central Africa and causes a chronic form of trypanosomiasis, whereas *T. b. rhodesiense* is prevalent in east and southern Africa and causes an acute form of the disease. *T. b. brucei* is not infective to humans, but instead infects wild and domestic animals causing a disease termed nagana (Brun et al., 2010).

The parasite is transmitted to the mammalian host by the bite of an infected tsetse fly (*Glossina* genus) (Jordan, 1993). Upon transmission from the fly into the bloodstream of the human host, the parasite lives and divides, with the first signs of infection being fever and joint pain. Currently, treatment is available for this stage of trypanosomiasis, however, it is often undiagnosed and in turn untreated (Doua et al., 1996). If left untreated, the parasite load increases in the infected individual and eventually crosses the blood-brain barrier and infects the central nervous system causing symptoms such as disturbance in sleep patterns and confusion (Buguet et al., 2006; Kennedy, 2006). Trypanosome infection is always fatal if left untreated (Brun et al., 2010). Treatment currently available for this stage of the disease is toxic, killing up to 10% of patients and requires high doses typically delivered by intravenous administration over several days to be effective (Schmid et al., 2005).

According to the World Health Organization, African sleeping sickness is considered a neglected tropical disease and remains an important public health issue (Brun et al., 2010). As

aforementioned, treatments currently available for African sleeping sickness are toxic and hard to administer, therefore, identification of potential drug targets to develop alternative therapeutic options for treatment of trypanosomiasis is a priority of research. Current *T. brucei* research, however, is not only focused on elucidating a treatment for disease, but also understanding the cell biology of the organism, including cell structure, organelle positioning, protein trafficking and cell division (Matthews, 2005) – these efforts may identify new targets for therapeutic development.

I. LIFE CYCLE OF *T. BRUCEI*

T. brucei spends its life in two distinct environments: the tsetse fly or the mammalian host. The nutrient availability and overall environments are different in the host and vector, requiring the organism to adapt to new biological niches as it transverses through the various life cycle stages (Figure 1.1).

Upon the bite of an infected tsetse fly, infective metacyclic *T. brucei* differentiate into long slender bloodstream forms (BSF) where they proliferate extracellularly in the bloodstream of the mammalian host. In the glucose rich bloodstream, metabolism is accomplished solely through glycolysis, as BSF parasites harbor a mitochondrion that is cryptic and not fully functional (Priest and Hajduk, 1994). The surface of BSF parasites is arrayed with glycophosphatidylinositol (GPI)-anchored variant surface glycoproteins (VSGs) that aid in evading the immune response of the host (Horn, 2001). Antigenic variation of the VSGs is utilized by the parasite, which has a repertoire of > 1000 VSG genes that it can switch periodically, to avoid death by the adaptive host immune response (Taylor and Rudenko, 2006).

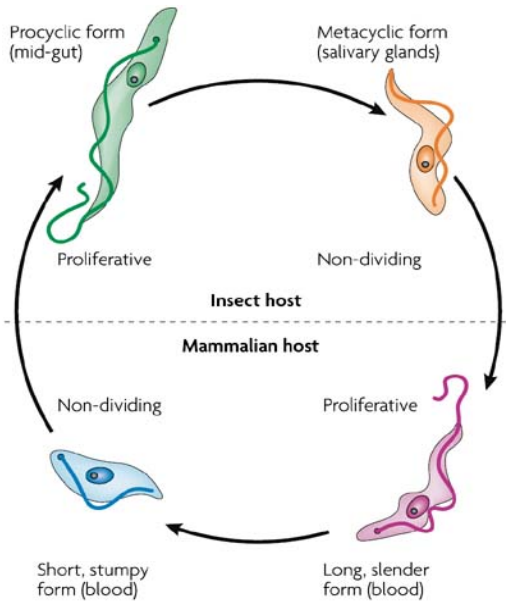

Figure 1.1 Simplified life cycle of *T. brucei*.

Long slender parasites proliferate in the mamammalian bloodstream. In preparation for life in the tsetse fly, BSF parasites differentiate into non-proliferative short stumpy form. Once taken up by the bite of a tsetse fly, the parasites morph into procyclic form parasites and rapidly divide in the midgut of the fly. To complete the life cycle, the parasites transition into non-dividing metacyclic forms, residing in the tsetse fly salivary gland. The parasites begin the life cycle again when they are transmitted back into the bloodstream of a mammalian host. (Image from: A fatty-acid synthesis mechanism specialized for parasitism. Lee, SH, Jennifer L. Stephens & Paul T. Englund *Nature Reviews Microbiology* 5, 287-297 April 2007. Permission granted for use of figure.)

Once BSF parasites reach a high density in the blood, a portion of the cells differentiate into stumpy form parasites in preparation for the bite of a tsetse fly (Vickerman, 1985, 1965). The mechanisms controlling the differentiation of BSF to stumpy form parasites are not clearly understood. Experimentally it has been determined, however, that long slender forms produce a

stumpy induction factor (SIF) to signal the switch. The identity and mechanism of SIF is currently unknown (MacGregor and Matthews, 2010).

As BSFs transition to stumpy forms many changes occur in the biology of the parasite. Stumpy forms are non-proliferative, cell cycle arrested, and have a more active mitochondrion to prepare them for the low glucose environment that they will soon encounter in the fly (Bass and Wang, 1991). Stumpy forms are arrested in the G1 stage of the cell cycle, making certain that changes that occur during transmission into the tsetse fly are coordinated with the resumption of the cell cycle (Ziegelbauer et al., 1990). The protein surface coat on the stumpy form remains the same as that of the long slender form, VSG, and is shed and replaced only after taking up residence in the tsetse fly (Matthews, 2005).

After a fly consumes a blood meal from an infected host, the stumpy form parasites transition to procyclic forms (PF) in the midgut of the fly. A heterogeneous population of trypanosomes consisting of BSFs and stumpy forms is consumed in the initial blood meal. Stumpy form parasites are able to survive the stresses in the harsh environment of the tsetse fly, while slender BSFs are not (Nolan et al., 2000). Short stumpy forms have developed mechanisms to adapt to the abrupt environmental, nutrient and temperature changes. Until 2009, the trigger that cued stumpy forms to switch to PFs remained elusive. However, it is now known that stumpy forms express a family of proteins, proteins associated with differentiation (PAD), that can sense the drop in temperature from the mammal (37°C) to the tsetse fly (20°C) which triggers the switch to the procyclic form parasite (Dean et al., 2009).

PFs rapidly divide in the tsetse fly gut displaying gross biological differences, including mitochondrial development and organelle positioning, compared to BSFs and short stumpy forms. These differences allow for survival in the glucose deplete environment of the tsetse fly.

To this end, PF parasites exhibit an active mitchodrion with components required for a functional Krebs cycle and electron transport chain. This elaboration of metabolic pathways permits PFs to metabolize amino acids, rather than solely glucose, for ATP production (Priest and Hadjuk, 1994).

During differentiation between stumpy forms and PFs, the VSG surface coat is replaced by new set of surface proteins called procyclins (Roditi and Clayton, 1999). Procyclins, like VSGs, are GPI anchored (Roditi et al., 1989). These surface proteins are characterized by their internal repeat motifs which consist of either repeats of glutamic acid-proline called EP, or repeats of 6 peptides (gly-pro-glu-glu-thr) termed GPEET (Roditi and Clayton, 1999). After PFs have sufficiently proliferated in the midgut, a portion of the parasites move to the salivary gland where they differentiate into the proliferative epimastigote form and attach to the salivary gland wall through changes made in their flagellar structure (Van Den Abbeele et al., 1999; Sharma et al., 2009). In preparation for infection of mammals, the parasites mature through one last developmental stage, the metacylic form, in which the surface coat is switched back to VSG and division is arrested (Van Den Abbeele et al., 1999). After differentiating to metacyclics, the parasites are prepared to infect a new host and repeat the life cycle. The process for PF parasites to progress to the metacyclic stage and become competent to infect a mammal takes ~3-5 weeks (Fenn and Matthews, 2007).

II. CELL STRUCTURE AND BIOLOGY

Trypanosomes are single celled protozoa classified as kinetoplastids. *T. brucei*, are microscopic and dependent on life cycle stage, range in size from 12-35 μM (Rotureau et al., 2011). The cell body of the parasite is organized in such a fashion that as it progresses through

the life cycle, single copy organelles of the cell (flagellar pocket, flagellum, kinetoplast, mitochondrion, and nucleus) can divide and differentiate efficiently (Matthews, 2005). An elongated and highly polarized microtubule cytoskeleton defines the shape of the parasite (Robinson et al., 1995). (Fig.1.2)

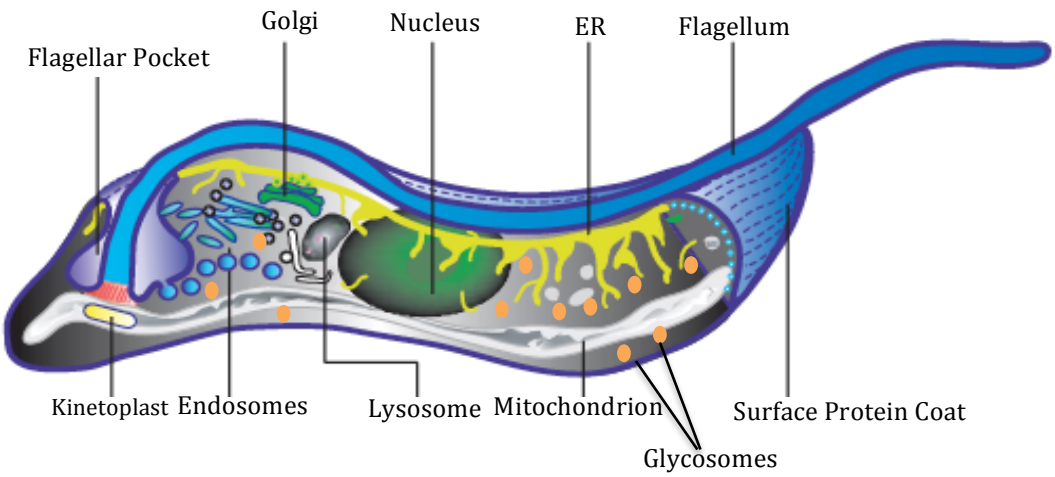

Figure 1.2 Schematic representation of the cellular organization of *T. brucei.*

T. brucei contain typical organelles found in eukaryotic cells. To note, *T. brucei* also contain unique organelles including the kinetoplast, flagellar pocket and glycosomes. (Adapted from: Overath, P. and Engstler, M. 2004. Endocytosis, membrane recycling and sorting of GPI-anchored proteins: *Trypanosoma brucei* as a model system. Molecular Microbiology **53**: 735-744. Permission granted for use of figure.)

A single flagellum arises out of the basal body near the posterior end of the cell and extends a short distance past the length of the cell (Ralston et al., 2009). *T. brucei* rely on their flagellum for motility, attachment to the tsetse fly salivary gland prior to transmission, and for a wide array of other cellular functions including proper cell division. The flagellum consists of a conventional axonemal structure (9+2 arrangement of microtubules) plus an associated paraflagellar rod, which contributes further to the motility of the organism (Vickerman, 1962;

Bastin and Gull, 1999). This organelle remains in close proximity to the cell via the flagellar attachment zone (FAZ), which includes a group of cytoskeletal and membranous attachments (Portman and Gull, 2010). Also important in the flagellar structure is the flagellar pocket, an invagination formed at the site where the flagellum emerges from the basal body (Ralston et al., 2009). The flagellar pocket is the only site of endocytosis or exocytosis for *T. brucei* (Overath and Engstler, 2004; Morgan et al., 2002).

All kinetoplastida contain a single mitochondrion. The mitochondrion function and development in *T. brucei* varies greatly, dependent on life cycle stage (Van Hellemond et al., 2005). *T. brucei* harbor a single elongated mitochondrion that runs from the posterior to the anterior of the cell. In BSF parasites the mitochondria is a simple tubular structure containing no cristae, as the parasite relies solely on glycolysis for ATP production (Opperdoes and Borst, 1977). PF parasites, however, have an active mitochondrion with components of the Krebs cycle and the electron transport chain being present and active to facilitate ATP production (Besterio et al., 2002).

Located in the mitochondrion of the cell is an organelle known as the kinetoplast. The kinetoplast is composed of the mitochondrial DNA (kDNA), arranged as a network of concatenated circles (Klingbeil et al., 2001). The kDNA consists of thousands of minicircles (0.5-10kb) and only 40-50 maxicircles (20-40kb) that encode mitochondrial gene products such as rRNAs and respiratory chain subunits (Van Hellemond et al., 2005). The expression of mitochondrial genes is unconventional, though, in that before the mRNA is competent to express a functional protein, it must be edited through the activity of minicircle encoded guide RNAs (Madison et al., 2002).

The position of the kinetoplast changes as the cell moves through the various life cycle stages. In BSF parasites, the kinetoplast is close to the posterior end of the cell whereas the kinetoplast is located midway between the cell nucleus and posterior end in PF parasites (Matthews, 2005). The repositioning of the kinetoplast is the most obvious morphological difference between the different life cycle stages of the parasites. The exact cellular mechanism of this repositioning is not clearly understood, but the correct positioning must occur for proper cell division in each life cycle (Matthews et al., 1995).

Another unique aspect of trypanosome cell structure is the sequestration of select metabolic enzymes in small organelles called glycosomes (Opperdoes and Borst, 1977). These organelles are abundant in the cell and share some similarity in content, structure, and biosynthesis to peroxisomes from other organisms (Parsons, 2004). The proteins housed in these small membrane bound organelles vary throughout the life cycle of the parasite as nutrient availability changes (Michels et al., 2006).

Glycosomes

Opperdoes and Borst (1977) first discovered glycosomes in *T. brucei*, revealing in BSF parasites these organelles housed nine glycolytic enzymes. The original discovery of these microbodies by Opperdoes et al. led to the name glycosome, to designate a unique organelle in the parasite (Parsons, 2004). Similar to peroxisomes in other organisms, glycosomes are bound by a single phospholipid bilayer and contain no DNA. Early studies revealed that the contents found in glycosomes were similar to those of peroxisomes in other organisms (Opperdoes, 1984). The hallmark peroxisomal enzyme found in other organisms is catalase, and only a few of the trypanosomatid species contain this enzyme in their glycosomes (Soares and De Souza,

1988). Glycosomal matrix and membrane proteins are imported post-translationally by a family of proteins called peroxins (PEX) (Moyersoen et al., 2004). Several peroxins have been identified in trypanosomes and are similar in sequence to yeast and human PEX proteins (Lorenz et al., 1998; Furuya et al., 2002; Guerra-Giraldez et al., 2002), .

Biogenesis of glycosomes in *T. brucei* is similar to the biogenesis of peroxisomes in other organisms (Moyersoen et al., 2004). As the parasites encounter highly different environmental conditions in each life cycle stage, the contents of the glycosomes must change to adapt to metabolic needs. When conditions change, as in the switch from the vector to the host, the contents in old glycosomes are no longer useful. Therefore, these glycosomes are degraded and replaced. This turnover process is a special form of autophagy, called pexophagy (Monastryska and Klionsky, 2006). Pexophagy is an important process for the cell during differentiation from BSFs to short stumpy forms. The process is more robust and rapid as the parasites transition from stumpy forms to PFs, reflective of a major change in environment (Herman et al., 2008). This remodeling event ensures that the metabolic contents of the glycosome are most optimally suited for the current environmental conditions.

Function of Glycosomes in BSF Parasites

Glycosomes of BSF parasites contain ~90% glycolytic enzymes, while the percentage of glycolytic enzymes in PF glycosomes is only 40-50% (Michels et al., 2006). One main postulation for the compartmentalization of glycolytic enzymes in BSF is the inability of *T. brucei* hexokinase (HK) and phosphofructokinase (PFK) to be inhibited by their products (Nwagwa and Opperdoes 1982; Cronin and Tipton, 1985; Lopez et al., 2002). Most HKs and PFKs, in other organisms, are tightly regulated by their products, or other metabolites, to prevent accumulation of glycolytic intermediates, which can be lethal to the cell (Haanstra et al., 2008).

Computer modeling to determine how glycolytic flux would be affected in BSF cells lacking compartmentalization of glycolytic enzymes revealed that steady-state glycolytic flux would not be altered, but that there would be a toxic accumulation of hexose-phosphates in the presence of glucose (Bakker et al., 1997). The cell, therefore compartmentalizes the glycolytic enzymes to prevent this toxic accumulation (and coincident depletion of cellular ATP) and to control the enzyme activity of HK and PFK (Bakker et al., 2000). The conclusion from this study and other studies reveal that glycosomes are essential for survival of BSF parasites (Guerra-Giraldez et al., 2002; Furuya et al., 2002).

The first seven enzymes of glycolysis are housed in BSF glycosomes to accomplish the conversion of glucose to 3-phosphoglycerate. The remaining steps of the pathway are accomplished in the cytosol (Opperdoes and Borst, 1977). Consumption and production of ATP and NADP by glycolysis are balanced within the glycosome and net ATP is produced in the cytosol of the cell, from the overall conversion of glucose to pyruvate (Opperdoes, 1987; Hammond et al, 1985; Hammond and Bowman, 1980).

Not only are glyocomes essential for BSF survival, correct localization of enzymes to this compartment is vital. Several studies have revealed that mislocalization of glycolytic enzymes is detrimental to the parasite (Blattner et al., 1998). One glycolytic enzyme, phosphoglycerate kinase (PGK) has two major isozymes, one that is expressed in the BSF (PGKg) which localizes to the glycosome, and one that is mainly expressed in PF (PGKc) which resides in the cytosol. BSF parasites were rapidly killed when PGKg lacking a glycosomal targeting signal or PGKc were expressed. These studies, among others, reveal that glycosomal compartmentalization of certain enzymes are necessary for survival of the cell (Blattner et al., 1998 and Helfert et al., 2001).

Function of Glycosomes in PF Parasites

PF parasites, unlike BSF, do not live in a glucose rich environment, but rather proline and threonine are the main carbon sources available in the insect vector. The cells do, however, prefer glucose as their main carbon source if it is available (Lamour et al., 2005). *In vitro* studies reveal that in glucose depleted media, PF parasites increase the rate (up to 6-fold) of proline consumption in the mitochondria for ATP production (Lamour et al., 2005).

Growth rates of PF in glucose deplete media are not impacted by the absence of glucose. This observation led to the conclusion that glycolysis is not essential in PF parasites, and in turn compartmentalization of glycolytic enzymes is dispensable (Lamour et al., 2005). In continued experiments, RNAi of PEX14, an essential protein for glycosome formation, was only lethal to PFs in the presence of glucose, which further confirmed that glycosomes are essential for PF survival only in the presence of glucose (Furuya et al., 2002).

Unlike BSF glycosomes which house mostly glycolytic enzymes, glycosomes in PF parasites contain enzymes important in other metabolic pathways including the pentose-phosphate pathway, purine salvage, β oxidation of fatty acids and biosynthetic pathways for pyrimidines, ether-lipids and squalenes (Michels et al., 2006).

III. GLYCOLYSIS IN *T. BRUCEI*

BSF parasites rely solely on glycolysis for ATP production, whereas, PF parasites utilize the breakdown of proline and threonine to generate ATP. The glycolytic pathway, however, does play a role in PF biology, as RNAi of glycolytic enzymes triggers a change in surface molecule expression (Morris et al., 2002). Also, rapid inhibition of glycolytic enzymes can be

lethal to PF parasites (Morris et al., 2002; Drew et al., 2003). Therefore, glycolysis is an essential metabolic pathway in BSF and PF *T. brucei*.

Hexokinase

Hexokinase (EC 2.7.1.1), the first enzyme in the glycolytic pathway, catalyzes the transfer of the γ phosphoryl group from ATP to glucose to yield glucose-6-phosphate and ADP. Although hexokinase is found in most living organisms, the enzyme is divergent across species with ~34% of the amino acid residues generally conserved in all members of the hexokinase family. This strong conservation points to the biological relevance of these residues (Kuser et al., 2000). Many organisms express more than one hexokinase and maintain pools of hexokinase containing several isoenzymes of different molecular mass and kinetic activity (Cardenas et al., 1998).

Sacchromyces cerevisiae expresses two hexokinase isoenzymes, PI and PII, which display 76% amino acid similarity (Kopetzki et al, 1985; Frohlich et al., 1985). The crystal structure of PII, similar to the structure of many other hexokinases, revealed that the molecule is distinctly folded into two domains: the large and small domains. The two domains are separated by a deep cleft where the active site residues are found. Many of the binding site residues in the cleft are strongly conserved across species (Kuser et al., 2000).

Catalytic base of hexokinase

The catalytic base of yeast HxkII has been identified as aspartic acid 211 (Asp 211) through tertiary structure determination and experimentation (Anderson et al, 1978.; Bennet and Steitz, 1980). The residue promotes the nucleophilic attack of glucose's 6-hydroxyl group on the γ-

phosphate of ATP followed by the transfer of γ-phosphate group to glucose (Jones et al., 1991). Asp 211 has also been determined to play an important role in glucose binding by hydrogen-bonding to OH groups of the sugar. Catalytic activity is lost when Asp 211 is substituted with another amino acid residue, however, high-affinity sugar binding is retained (Kraakman, et al., 1999). The catalytic aspartic acid residue is conserved in other hexokinases including human pancreatic β-cell glucokinase (Asp 205) (Charles et al., 1994; Gidh-Jain et al., 1993) and *Trypanosoma brucei* hexokinase 1 (Asp 214) (Morris et al., 2006) .

T. brucei Hexokinase 1 and Hexokinase 2

T. brucei expresses two hexokinases, TbHK1 and TbHK2, which are 98% identical at the amino acid level, with differences found in the C-terminal end of the proteins (Morris et al., 2006). TbHK1 and TbHK2 are arranged in tandem on chromosome 10, are both expressed in BSF and PF parasites (Berriman et al., 2005; Colasante et al., 2006). RNAi and knockout experiments revealed that TbHK1 and TbHK2 are important for both life cycle stages of the parasite. Both HKs are essential to BSF parasites, while PFs with a TbHK2 double allele knockout are viable, though distinct morphological characteristics were noted (Chambers et al., 2008; Albert et al., 2005; Morris et al., 2006). Current unpublished work suggests that TbHK2 may play a role in glucose sensing in BSF as evidenced by its localization to the flagellum (Joice and Lyda, unpublished observation). To date, PF TbHK1 double allele knockouts have not been successfully generated (M.Morris, unpublished observation). The essentiality of the TbHKs in BSF parasites and their small percentage of similarity to human glucokinase (~30% similar), make them prime drug target candidates.

A crystal structure has not been solved for either *T. brucei* hexokinase, however, both proteins have been modeled on yeast PII (Morris et al., 2006). The modeling revealed that the TbHKs, similar to yeast HKs, are composed of two domains separated by a deep cleft containing the active site (See Figure 1.3), and differences between the two almost identical enzymes lie within the cleft. Several important active-site residues are located in this cleft along with the amino acids responsible for binding glucose (Morris et al., 2006). Additionally, the catalytic base is located in the cleft (Kuser et al., 2000). Modeling also revealed that the unique C-terminal peptide sequence found in TbHK2 may not allow for conformational changes needed for catalytic activity, or may change the position of the catalytic base and prevent activity (Morris et al., 2006).

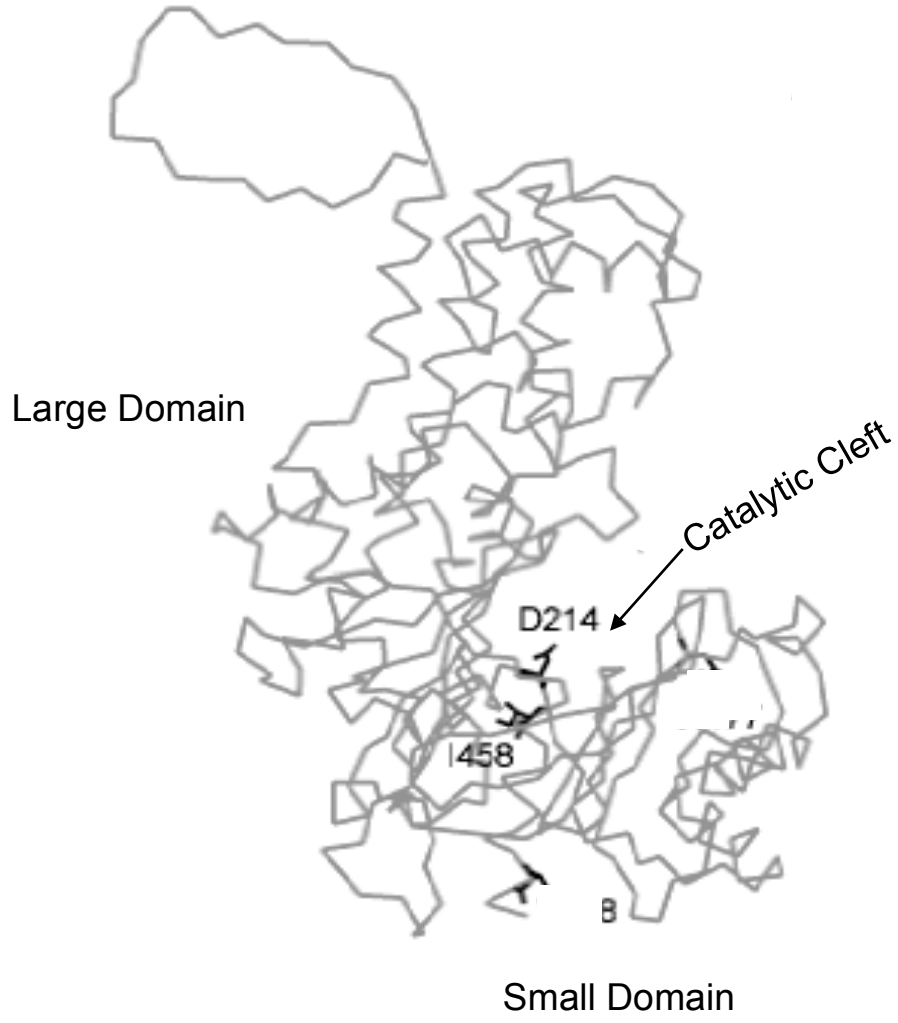

Figure 1.3 *T. brucei* hexokinase modeled on yeast hexokinase II.

DS-Modeler- generated structure of *T. brucei* hexokinase. The protein is comprised of a large domain and small domain with a deep cleft containing catalytic residues between the two domains. Catalytic residue, D214, is highlighted. The N-terminal signal structures remain unstructured. (Image adapted from: Morris, M. et al. 2006. Activity of a Second *Trypanosoma brucei* hexokinase is controlled by an 18-Amino-Acid C-Terminal Tail. Eukaryotic Cell **5**: 2014-2023. Permission granted for use of figure.)

Characterization of TbHKs

Initial characterization of *T. brucei* hexokinase (*Tb*HK) revealed that unlike most other eukaryotic HKs it is not regulated by Glucose-6-phosphate or ADP (Misset et al., 1986). Another early observation found that TbHK can use a myriad of substrates as its phosphoryl donor including ITP, UTP, CTP, GTP or ATP (Nwagwu and Opperdoes, 1982). Early research also indicated instead of existing as a monomer or dimer in the cell, TbHKs exist as multimers, containing up to six subunits (Misset et al., 1986).

Continued characterization of the enzymes revealed that the TbHKs localize to the glycosome, which is not suprising in that they both contain peroxisomal targeting sequences (PTS) at the N-terminal end of the protein. Recent studies suggest that TbHK2 may also localize to the flagellum (Coley and Lyda, unpublished results). Of note, rTbHK2 lacks detectable HK activity, while rTbHK1 does display hexokinase activity (Morris et al., 2006). To this end, it is speculated that native HK activity is obtained from a mixture of TbHK1 and TbHK2 monomers because the molecular mass of the protein from which native HK activity was obtained was reflective of a hexameric oligomer (Morris et al., 2006). The exact composition of the hexamer in its native state, however, is not currently known.

Regulation of Hexokinase Activity

As mentioned previously, TbHKs are unlike most eukaryotic hexokinases that are regulated via feedback inhibition by their products. Regulation of TbHKs is not accomplished in a conventional manner, but rather through non-conventional ways such as the oligomerization state of the enzyme or inhibition by fatty acids found in the glycosome (Chambers et al., 2008; Morris et al., 2006).

IV. GENE EXPRESSION

In order for a cell to survive under changing conditions, RNA is continually transcribed to carry on basic cell functions such as reproduction, growth, repair and regulation of metabolism. Most organisms control gene expression at the level of transcription initiation. However, there are other control points that cells often employ to regulate transcription including RNA stability and transport, RNA processing and translation. *T. brucei* regulate almost all gene expression post-transcriptionally, due to the polycistronic arrangment of the genome (Clayton, 2002).

Polycistronic Transcription

The genome of *T. brucei* is 26 Mb and consists of 11 chromosomes coding for ~9,000 genes (Berriman et al., 2005). The annotation of the *T. brucei* genome in 2005 confirmed that protein-coding genes are arranged in large polycistronic units, displaying a unique arrangement of gene structure for a eukaryotic parasite (Berriman et al., 2005). Transcription, therefore, is accomplished in a polycistronic fashion with RNA Polymerase II transcribing up to several hundred contiguous genes (50-200kb) from one polycistron. Unlike operons in bacteria that encode functionally related genes, genes that are co-transcribed in *T. brucei* are not necessarily functionally related (Haile and Papadopoulou, 2007).

The entire genome contains ~200 total bi-directional polycistronic units (Nilsson et al., 2010). Genes are transcribed in one direction (from the same strand) within a polycistronic unit; however, neighboring units can display convergent or divergent transcription (Siegel et al., 2009). The region between two polycistronic units is called a strand switch region (SSR). In *Leishmania major,* a kinetoplast sharing 75% similarity in gene arrangement with *T. brucei*, the

SSRs have been shown to be initiation or termination sites for transcription with transcription starting between two divergent SSRs and terminating between convergent SSRs of polycistronic units (Martinez-Calvillo et al., 2003). It is thus hypothesized that *T. brucei* transcription start sites are also located at divergent SSRs (Siegel et al., 2009). Further, recent data provides evidence that the chromatin structure at the SSRs plays a role in RNA polymerase II transcription initiation (Siegel et al., 2009).

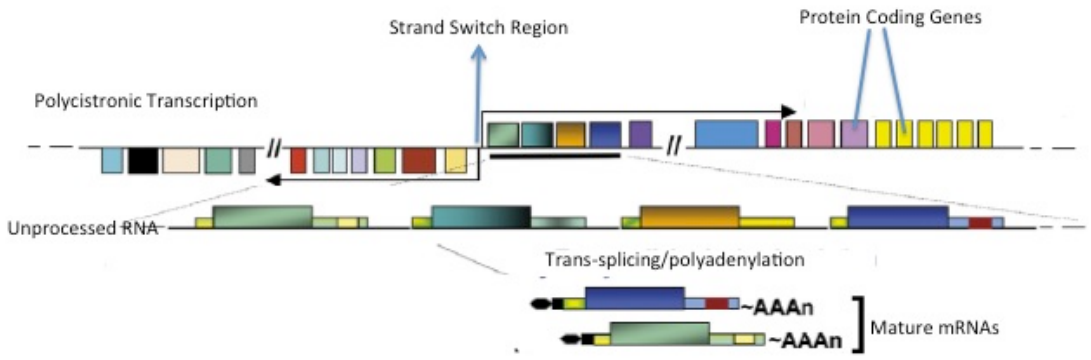

Figure 1.4 Diagram of Transcription/mRNA Maturation in *T. brucei*

T. brucei genes are arranged in bi-directional polycistronic units with transcription of up to several hundred genes occurring from one unit. Strand switch regions are located at divergent transcription sites. After transcription, the RNA is processed in a coupled *trans*-splicing/polyadenylation reaction to generate mature mRNA ready for translation. (Figure adapted from: Haile, S., and Papadopoulou, B.. Developmental regulation of gene expression in trypanosomatid parasitic protozoa. Current Opinion in Microbiology. 2007; **10:** 1-9. Permission granted for use of figure.)

One other proposed mechanism involved in transcriptional initiation in kinetoplasts is the presence of a modified thymine, called Base J (Bernards et al., 1984). This modified base was first discovered in the telomeric regions of expression sites of silenced VSGs in BSF parsites.

Base J is present in nuclear DNA specifically in inactive VSG expression sites. For this reason, it was initially thought that Base J played a role in gene silencing, however, studies have revealed that this is most likely not the role of Base J (Cross et al., 2002). Current data suggests that Base J is involved in some type of telomeric function, its exact role, though is yet to be discovered (Borst and Sabatini, 2008).

The polycistronic nature of transcription implies that gene expression is not regulated at the level of transcription initiation in *T. brucei* , but instead is accomplished almost exclusively in a post-transcriptional manner. To date only one RNA Polymerase II promoter has been identified and characterized from *T. brucei.* This promoter is responsible for driving expression of the genes for the spliced leader (SL) precursor RNA. Uncharacteristic of eukaryotic promoters, this promoter does not contain TATA or CCAAT boxes (Bayele, 2009).

Other promoters have also been identified specifically for genes encoding the surface proteins of both procyclic form (PARP) and bloodstream form (VSG) parasites (Clayton et al., 1990; Gottesdiener et al., 1991). Notably, these genes are transcribed by RNA polymerase I, which is usually responsible for transcribing ribosomal RNA genes in other organisms. With the exception of the few promoters identified, DNA sequences and proteins involved in initiation and termination of transcription are not well understood in *T. brucei* (Martinez-Calvillo et al., 2010). The lack of conventional RNA Polymerase II promoters and the mechanism of polycistronic transcription together suggest that transcription is primarily constitutive and gene levels are regulated following transcription. Indeed, the experimental data acquired to date supports this claim, with elements in the 3'UTRs of genes playing a central role in regulation (Hehl et al., 1994; Berberof et al., 1995; Hotz et al., 1995; Furger et al., 1997; Vassella et al., 2000).

Processing of mRNA

mRNA processing of individual genes occurs after initial transcription of the long array of genes from each polycistronic unit. mRNA processing in *T. brucei* is a coupled process entailing a 5' and 3' modification for the mRNA of each protein-coding gene. The first processing reaction is a *trans*-splicing event in which a conserved 39nt SL sequence is added to the 5' end of each protein-encoding RNA (Sutton and Boothroyd, 1986) providing a cap for the mRNA. Bioinformatic tools have been used to predict splice sites, which are U-rich polypyrimidine tracts that precede AG acceptor sites (Clayton, 2002). Recently, these sequences were confirmed experimentally in work published by Nilsson et al. (2010) in which high throughput sequencing was used to identify splice sites for almost all of the protein coding genes in *T. brucei*. In these experiments, 2500 alternative splice sites were also identified. The alternative splice sites identified are specific to one of the life cycle stages, revealing that alternative splicing may be a means of regulation for the parasite.

Addition of the SL to the 5' end of an mRNA is coupled to 3' polyadenylation. This is a coupled reaction, meaning the splice site for a particular gene influences the choice of the polyadenylation site for the gene directly upstream (LeBowitz et al., 1993). Unlike higher eukaryotes, there is no conserved polyadenylation signal sequence in *T. brucei*, therefore prediction of polyadenylation sites can only be estimated by distance from the downstream *trans*-splicing event (Clayton, 2002). In the past year, however, polyadenylation sites for almost 6,000 *T. brucei* genes have been identified using RNA-seq technology (Siegel et al., 2010). The data from this effort revealed that many genes contain several functional polyadenylation sites. The importance of multiple polyadenylated species for the same transcript has not yet been determined.

Post-transcriptional Regulation

The changing environments that *T. brucei* encounters during its life requires plasticity in gene expression so that genes can be expressed at different levels as the environment and nutrient availability changes. Most of the genes in trypanosomes are initially transcribed at the same levels from a polycistron (Martinez-Calvillo et al., 2010). The coupled process of pre-mRNA processing suggests that regulation does not occur during this event, as two unrelated genes that are located in tandem in a polycistron are processed in the same coupled reaction yet often exhibit distinct expression profiles (Kabani et al., 2009). Further, several genome wide studies have recently revealed that only a modest number (between 2-10%) of all *T. brucei* genes are regulated at the RNA level (Siegel et al., 2009; Brems et al., 2005; Koumandou et al., 2008; Jensen et al., 2009; Kabani et al., 2009).

Post-transcriptional regulation can occur at several levels in the cell; mRNA stability and degradation, translational efficiency, and protein processing, modification and stability (Haile and Papadopoulou, 2007). These post-transcriptional mechanisms of regulation can affect splicing, transport, stability, localization and translation of mRNAs (Ouellette and Papadopoulou, 2009). There are many factors involved in controlling these regulatory processes including trans-factors such as RNA binding proteins and small RNAs (Clayton and Shapira, 2007). The trans-factors act on cis sequences usually found in the 3'UTRs of mRNAs (Ouellette and Papadopoulou, 2009).

mRNA Stability and Regulation

One post-transcriptional mechanism through which trypanosomes regulate gene expression is RNA stability**.** There are two different ways that mRNA degradation is accomplished in the cell. An mRNA can be degraded through deadenylation by a poly A

nuclease followed by degradation in the 3' → 5' direction by the exosome (Buttner et al., 2006). An alternative route used in cells is the removal of the 5' cap by a protein complex followed by mRNA degradation in the 5' → 3' direction (Parker and Song, 2004). Trypanosomes express the proteins necessary for both deadenylation and decapping, and both activites have been detected in trypanosome lysates and deadenylation has been detected *in vivo* (Milone et al., 2002; Haile et al., 2003). Sequences involved in stabililizing mRNA have been identified in the 3'UTRs of several trypanosome genes (Hehl et al., 1994). Regulatory proteins or other trans elements that bind to the 3'UTRs to confer mRNA stabilization have not yet been identified (Clayton and Shapira, 2007).

3'UTR Sequences Involved in Regulation

Sequences have been identified in the 3'UTRs of various *T. brucei* genes that regulate gene expression by controlling mRNA stability and degradation or by mediating translational efficiency (Clayton, 2002; Haile and Papadopoulou, 2007).

The most efficient way to identify specific sequences of the 3'UTR involved in regulation is to use a reporter gene system. After either stable or transient transfection of reporter gene constructs into pertinent cell lines, mRNA amount, protein expression and mRNA stability can be quantified (Clayton, 2007). This technique has been used to identify regulatory regions in many *T. brucei* 3'UTRs.

The EP procyclin 3'UTR is one of the most well characterized *T. brucei* UTRs. Hehl et al. (1994) identified both a 16-mer and a 26-mer that are involved in regulating the expression of this protein. The 16-mer enhances translation in PFs and the 26-mer is necessary for the instability of EP procyclin mRNAs in BSFs. Though these are two of the most well

characterized sequences, many others involved in regulation of gene expression have been identified in trypanosome 3'UTRs. In some genes, however, it has been challenging to narrow down specific regions and mechanisms involved in regulation (Clayton and Shapira, 2007).

Coordinated gene expression

Several recent publications examining genomic analysis during different life cycle stages of the parasite have revealed that post-transcriptional regulation is accomplished in a coordinated fashion (Kabani et al., 2009; Jensen et al., 2009; Queiroz et al., 2009). These studies revealed clusters of coordinated gene expression, with many of the clusters containing genes with a variety of functions; however, some of the genes contained within the cluster were functionally related. It is postulated that there may be proteins that recognize a group of mRNAs containing the same sequences in their 3'UTRs, conferring stability on the group of mRNAs in a coordinated way (Ouellete and Papadopoulou, 2009). The set of mRNAs regulated together is referred to as a regulon. The complete and detailed mechanisms of coordinated regulation and cellular components involved are not yet clearly understood. Further characterization of the 3'UTRs of the genes found in these regulons may reveal sequences that are involved in global regulation of gene expression in *T. brucei*.

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CHAPTER TWO

DIFFERENTIAL POLYADENYLATION INFLUENCES GENE EXPRESSION IN THE AFRICAN TRYPANOSOME IN RESPONSE TO ENVIRONMENTAL CUES

Heidi C. Dodson, Andrew C. Sayce, Meredith T. Morris, and James C. Morris

Department of Genetics and Biochemistry

Clemson University

Clemson, SC 29634

ABSTRACT

Cellular responses to environmental changes include altered gene expression of metabolic genes. In *Trypanosoma brucei*, regulatory elements in the 3'UTRs dictate transcript abundance and protein expression. Through deep sequencing, multiple transcripts generated by differential polyadenylation have been identified for most genes. Here, we have described how abundance and expression of seven different transcripts for *T. brucei* hexokinase 1 (*TbHK1*) are influenced by polyadenylation site in a carbon source-dependent fashion. Under standard growth conditions, relative expression of the *TbHK1* 3'UTR lengths was similar in procyclic form and bloodstream form parasite, though overall expression was higher in the bloodstream form. Of the seven tested, steady-state abundance of transcripts bearing one 3'UTR (1339 nt) was increased more than 9-fold in the presence of glucose, and two (1352 nt and 1086 nt) were reduced by more than 10-fold. Translation for all lengths, as measured in a chloramphenicol acetyl transferase reporter assay, was either down-regulated or unchanged by glucose. TbHK1 expression was also dynamic as a result of growth in glycerol-based media, with the steady-state

levels of two transcripts (bearing either the 1331 nt or 1034 nt 3'UTR) increased more than 20-fold while two (1352 nt and 1086 nt) were decreased more than 6-fold. Translation from the transcript bearing the 1331 nt 3'UTR was also increased ~2-fold, while other 3'UTRs either had no impact or attenuated expression in response to glycerol. Notably, steady-state transcript levels of the single polyadenylated *TbHK2* species also responded to carbon source, with a ~3-fold increase in the presence of glucose. Translation of *TbHK2* was nearly undetectable in the absence of glucose or glycerol, but similar was expressed at similar levels in the presence of either carbon source.

INTRODUCTION

Organisms that occupy multiple biological niches must metabolically adapt to different environments. *Trypanosoma brucei*, the protozoan parasite that is the causative agent of African sleeping sickness, alternates between a mammalian host and an insect vector. As they move from the mammalian bloodstream to the tsetse gut and migrate to the salivary glands (through tissues and the hemocoel/plasma, a migration that is poorly understood), the parasites are exposed to a variety of environmental cues. These include proteases and pH changes in the insect gut, fluctuating temperatures (the fly is a poikilotherm, so nighttime temperatures may be significantly less than day), and exposure to different small molecules, both as the bloodmeal is digested by the fly and during migration through fly tissues.

For a small molecule to serve as an effective "marker" for a particular environment, its availability must change from one niche to another. Glucose is an example of such a molecule. Short stumpy form parasites ingested by a feeding tsetse fly experience a rapid drop in glucose concentration, with the sugar in the blood nearly depleted in ~15 minutes (Vickerman, 1985). BSF parasites may also encounter changes in glucose concentrations. Both cerebrospinal fluid and blood glucose concentrations increase in febrile children (Kirviranta et al., 1995) (fever is frequently associated with trypanosomiasis), while mice infected with *T. rhodesiense* have decreased serum glucose (Moon et al., 1968).

In vitro, manipulation of glucose levels trigger changes in gene expression, supporting the hypothesis that the sugar may act as a key player in environmental sensing. For example, GPEET procyclin expression is regulated by mitochondrial enzymes in response to changes in glucose concentrations in the culture (Vassella et al., 2004). Cultured BSF can be triggered to express PF parasite characters by removal of glucose from the growth medium, even at 37^{o}C

(Milne et al., 1998). Last, RNAi silencing of glycolytic genes, including the TbHKs, disrupts the developmentally coordinated expression of surface molecules in PF parasites, suggesting a connection between the metabolic pathway and development (Morris et al., 2002).

Because the trypanosome genome is organized with protein coding genes arrayed in units transcribed into single polycistronic RNAs, post-transcriptional mechanisms serve as key regulators of gene expression control. Following transcription of the pre-mRNA polycistron, maturation of individual messages occurs in two coupled reactions, with trans-splicing adding a small capped RNA (the splice leader, SL) to the 5' end while cleavage and polyadenylation provide the maturation of the 3' end of the gene. As a result of this maturation process, which is common for all protein coding genes, gene regulation is usually mediated by elements found in message 3'UTRs. For example, the 3'UTRs of procyclins have been shown to regulate both mRNA levels and translation (Hotz et al., 1997). Elements involved in post-transciptional regulation have been identified, including a glucose and glycerol response element in the 3'UTR of GPEET procyclin (Vassella et al., 2004).

Hexokinase (HK), the first enzyme in glycolysis, catalyzes the phosphorylation of glucose to form glucose-6-phosphate. *T. brucei* strain 927 harbors two TbHK genes, *TbHK1* and *2*, that are encoded in tandem on chromosome 10 (Morris et al., 2002; 2006). Both genes are expressed in BSF and PF parasites, yielding polypeptides that are 98% identical (Morris et al., 2006; Colasante et al., 2006). While the putative 5'UTRs and ORFs of the genes are 98.5% identical at the nucleotide level, the 3'UTRs are dissimilar. One consequence of the differences in the 3'UTRs is that transcript maturation yields a different number of mature polyadenylated messages. Seven different polyadenylated *TbHK1* transcripts have been identified in a genome-wide analysis of transcript splicing and polyadenylation, while only one transcript for *TbHK2* has

been identified (Siegel et al., 2010). The molecular mechanisms behind the generation of the different species of *TbHK1* message are unknown; however, here we present evidence that the different transcript lengths are maintained at different abundance and have distinct expression patterns in response to environmental growth conditions. These findings suggest that differential polyadenylation of mRNA in *T. brucei* serves as a mechanism for gene regulation in the African trypanosome.

MATERIALS AND METHODS

Trypanosome growth and transfection - Procyclic form *T. brucei* 29.13, a 427 strain that expresses T7 RNA polymerase and the tetracycline repressor, were grown and maintained in SDM79 (Brun and Shonenberger, 1979) supplemented with 15% fetal bovine serum under standard growth conditions (29°C, 5% CO_2). For experiments performed in low glucose or glycerol supplemented media, SDM80 (Lamour et al., 2005) was prepared with 9% heat-inactivated dialyzed FBS and 1% heat-inactivated non-dialyzed FBS and, where noted, 10mM glycerol was added to the culture. Transient transfections (1×10^8 cells, 10μg DNA) were performed in 0.4 cm gapped cuvettes using an ECM 830 Electroporator (Genetronics, Inc., San Diego, CA). Briefly, cells were pelleted (800 x g, 10 min), washed, and then transferred to a cuvette with DNA and transfected (1700V, 100μs, 3 pulses, 200ms). Immediately after transfections cells were resuspended in SDM79, SDM80 or SDM80 supplemented with glycerol for 24 hours prior to RNA isolation, CAT assay or TbHK assay. (Please note that results of assays performed in SDM79 (6 mM glucose) yielded results that were essentially identical to those performed in SDM80 supplemented with glucose.)

Constructs - For all CAT reporter constructs, pGAPRONE (Furger et al., 1997) harboring a CAT reporter gene under the control of a procyclin promoter was used as the backbone for cloning of the respective TbHK 3'UTRs. In short, primers containing a *Bam*HI site in the forward primer and *Xba*I site in the reverse primer for cloning were designed to amplify the varying lengths of authentic *TbHK1* UTRs. The preexisting EP1 3'UTR in the parent pGAPRONE vector was removed by restriction enzyme digest and amplified UTRs were ligated in its place. All constructs were confirmed by DNA sequencing.

Quantitative Real Time PCR - Steady-state mRNA abundance of constructs bearing the authentic *TbHK1* UTRs was assessed by qRT-PCR using primers specific to the CAT reporter gene (F CAT: 5' GCGTGTTACGGTGAAAACCT 3' and R CAT: 5' ATCACA GACGGATGATGAA 3') which yields a 219 bp PCR product. Actin was used as the reference gene for all qRT-PCR experiments (F Actin: 5' GCCACGTATTTCCATCCATC 3' and R Actin: 5' CCTGAGCTTCATCACCAACA 3'). For qRT-PCR, a total of 5 $x10^6$ PF parasites were pelleted (800 x g, 10 min), washed in PBS and total RNA isolated (Aurum™ Total RNA Mini Kit, Bio-Rad, Hercules, CA) followed by DNase treatment on the purification column per the manufacturers instructions. A one-step qRT-PCR kit (iScript One-Step RT-PCR Kit with SYBR Green, Bio-Rad, Hercules, CA) was used for cDNA synthesis and amplification. qRT-PCR was performed on an iCycler iQ System (Bio-Rad, Hercules, CA) and resulting data was analyzed using the Pfaffl method (Pfaffl, 2001).

CAT Reporter Gene Assays - Chloramphenicol acetyl transferase (CAT) activity was assessed in lysates from transiently transfected cells in a 96 well plate format using a GENios

spectrophotometer (Tecan Group Ltd., Switzerland). Twenty-four hours post-transfection, cells were counted and hypotonically lysed. Assays were performed in 250 μL total volume containing final concentrations of 100 μM Acetyl-CoA (MP Biomedicals, Solon, OH), 100 μM chloramphenicol (MP Biomedicals, Solon, OH) and 0.5 μM DTNB (G Biosciences, Maryland Heights, MO). The assay scores the transfer of the acetyl group to one of the two hydroxyl groups on chloramphenicol. During this transfer, a thiol is released from the acetyl-CoA which reacts with 5,5-dithio-bis 2-nitrobenzoic acid (DTNB) to release 2-nitro-5-mercaptobenzoic acid (TNB), which then is measured by absorbance at 405nm.

Hexokinase Assays - To assess hexokinase activity from PF cells, lysate was prepared as described (Chambers et al., 2008). Hexokinase activity was measured in triplicate using a previously described coupled reaction (Morris et al., 2002).

RESULTS

The genome of the African trypanosomes is organized into polycistronic units that are transcribed into long RNAs that, through RNA maturation, yield functional transcripts. As a result of this unusual transcription mechanism, regulation of gene expression occurs primarily post-transcriptionally with 3'UTR sequences serving to regulate gene expression. Recently, Siegel et al. (Siegel et al., 2010) identified polyadenylation sites for 5,948 *T. brucei* genes using high throughput RNA sequencing technology (Siegel et al., 2010). *TbHK1* had seven distinct polyadenylation sites resulting in 3'UTR lengths of 1026, 1034, 1086, 1166, 1331, 1339 and 1352 nt (Fig. 2.1A). *TbHK2*, however, had only one polyadenylation site, yielding a 3'UTR of 353 nt. The relative abundance of the UTR lengths in PF parasites cultured in standard media

(SDM 79) was also reported, with 1339 nt being the most abundant (33% of the total) while the 1352 nt and 1166 nt species made up 17% (each) of the total. The remaining four lengths accounted for the rest, with each having an abundance of 8% (Sigel, 2010).

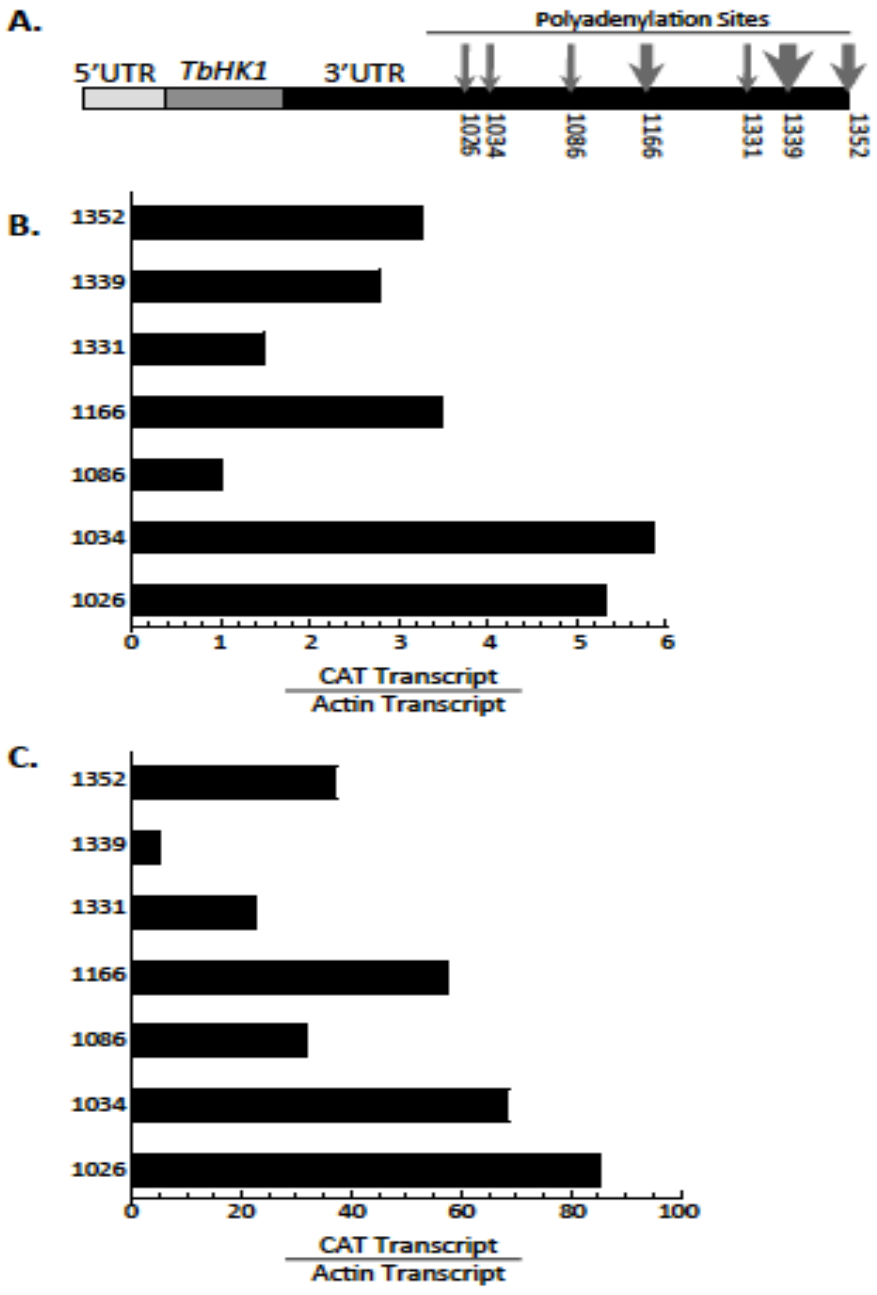

Figure 2.1 Polyadenylation influences *TbHK1* steady state abundance in PF and BSF parasites.

(A) Schematic representation of *TbHK1* depicting the location and abundance of polyadenylation sites for the gene (Siegel, 2010). The thickness of the arrow corresponds to the percentage of the UTR transcript detected in PF cells maintained in glucose-rich media: 1026, 1034, 1086 and 1331 (8% each), 1166 and 1352 (17%) and 1339 (33%). Relative abundance of a reporter gene bearing the different *TbHK1* 3'UTRs in PF (B) and BSF (C). Both cell lines were transiently transfected with a construct containing the respective UTR length downstream of the CAT reporter gene. qRT-PCR was used to measure CAT transcript abundance with actin transcript as a reference (Pfaffl, 2001).

TbHK1 *transcript abundance in both PF and BSF parasites is dependent on polyadenylation site.* The presence of multiple polyadenylated *TbHK1* transcript species, particularly in light of the single *TbHK2* species, suggested that the distinct UTRs might have a role in the regulation of *TbHK1*. To study the consequences of the different lengths of UTRs on steady state mRNA levels, PF and BSF parasites were transiently transfected with an expression vector containing a CAT reporter gene fused to the *TbHK* UTRs, followed by analysis of CAT transcript levels by qRT-PCR using primers to the CAT ORF (Fig. 2.1B and 2.1C).

In both PF and BSF parasites, the shorter UTRs typically yielded the highest relative abundance (compared to the reference gene actin), with the distribution of the relative transcript levels being remarkably similar between the two life cycle stages. However, there are notable exceptions to this trend. Unlike the PF cells, which maintain nearly twice as much 1339 nt 3'UTR transcript as the 8 nt shorter 1331 nt construct, BSF have higher steady-state levels of the 1331 nt 3'UTR than the 1339 nt length.

TbHK1 *3'UTRs influence both transcript abundance and gene expression in response to glucose depletion* - The diversity of 3'UTRs associated with the TbHK1 gene led us to consider the possibility that the process of message maturation, particularly polyadenylation, may be part of a

response to environmental cues. Using qRT-PCR, the transcript levels of CAT harboring the *TbHK1* 3'UTRs were quantified in PF cells that were either maintained in glucose-rich media or were shifted to low glucose media after transfection of the reporter. In response to glucose, steady state levels of 1339 nt 3'UTR reporter were increased more than nine-fold (Fig. 2.2A). Transcripts harboring other *TbHK1* 3'UTR lengths were less responsive to glucose, with the 1166 nt 3'UTR and 1026 nt 3'UTR in particular demonstrating little change in abundance. Transcript levels for constructs bearing the 1352 nt and 1086 nt 3'UTR were reduced, with the former reduced ~10-fold while the latter was reduced to undetectable levels.

To assess the consequences of the 3'UTRs on expression, CAT enzyme activity was monitored from cell lysate from transfectants. In the absence of glucose, expression from all constructs was detected, ranging from 3.22-8.5 units of CAT activity (Fig. 2.2B). However, culturing the transformed parasites in the presence of glucose had a marked impact on CAT expression. Expression of CAT from constructs harboring the two longest 3'UTRs (1352 and 1339 nt) was undetectable, while expression from other constructs was unaffected by the presence or absence of glucose (1086 nt, for example).

Glycerol as a carbon source alters TbHK1 *transcript abundance and expression in a 3'UTR-dependent fashion* - Glucose and glycerol have been identified as glycolytic substrates that can influence gene expression in PF *T. brucei.* Both compounds regulate GPEET procyclin expression, for example, through a single element in the 3'UTR (the GRE) (Vassella et al., 2004). To assess the impact of glycerol on TbHK1 expression and determine if particular 3'UTR lengths influence the response, both transcript steady state levels and expression were scored in transiently transfected parasites grown in glycerol using the CAT reporters described above.

While the over-all fold change in transcript abundance was greater after cells were shifted to glycerol containing media (as compared to those shifted to glucose (Fig. 2.2A)), there was a notable conservation of the pattern of transcript abundance. For example, constructs bearing the 1086 nt 3'UTR were once again reduced (as a result of growth in either glucose or glycerol), while 1034 nt 3'UTR had a greater fold-change in both glucose and glycerol than the 1026 nt 3'UTR constructs (Fig. 2.3A). This trend was true with one dramatic exception – constructs bearing the 1331 nt 3'UTR were increased more than 30-fold in response to glycerol, while the 1339 nt 3'UTR constructs were unchanged (Fig. 2.3A). This observation contrasts with the observed relative increase in the 1339 nt 3'UTR construct in response to glucose (Fig. 2.2A).

For the majority of the 3'UTRs, CAT expression was modestly altered as a result of culturing in the presence of glycerol (Fig. 2.3A). Expression from constructs bearing the 1026, 1034, and 1086 nt UTRs was repressed (1.45-2.7-fold), while the near complete repression of 1166 and 1339 nt 3'UTR-bearing constructs was similar to the loss of expression observed for the same constructs in response to glucose. Conversely, the expression from the 1331 nt 3'UTR construct was increased nearly 2-fold, and the 1352 nt 3'UTR was minimally enhanced (1.2-fold), though the maintenance of expression of the latter construct is in marked contrast to its complete repression in the presence of glucose (Fig.2.2B).

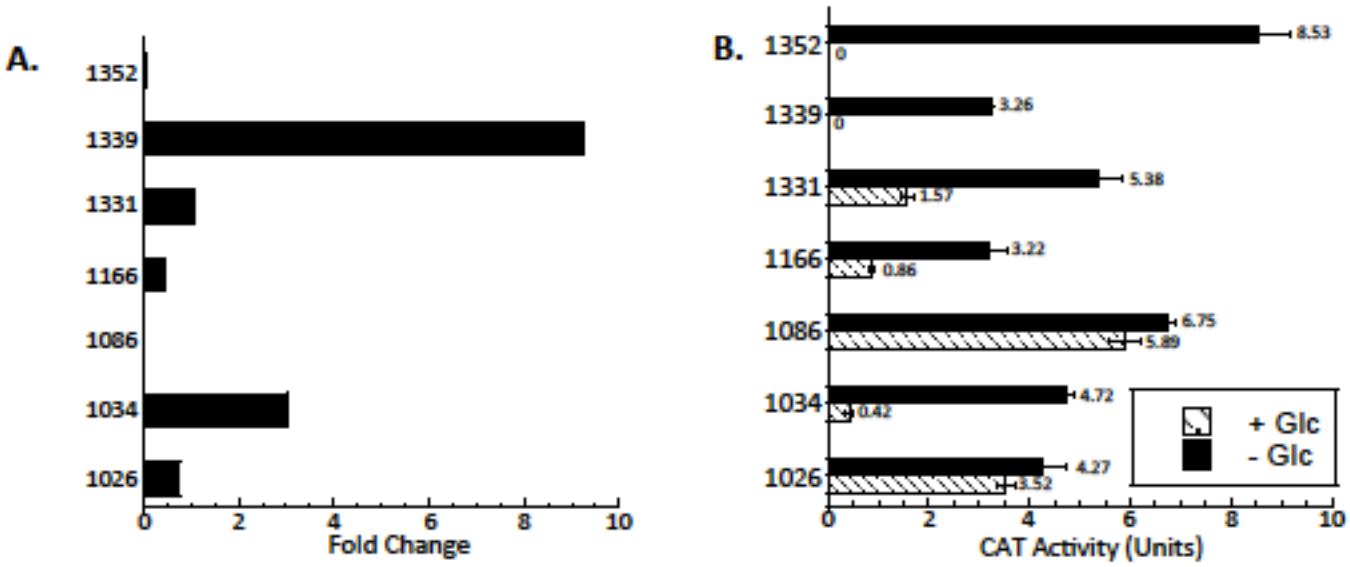

Figure 2.2 Transcript and expression levels of CAT bearing the seven different TbHK1 3'UTR lengths in glucose-rich media.

(A) Transcript levels measured from PF cells cultured in either glucose-rich or deplete media following transient transfection with constructs harboring the TbHK1 3'UTRs. Fold change was determined using the Pfaffl method with comparisons made between cells cultured in low glucose versus high glucose. A value equal to one indicates no change in transcript abundance. (B) Expression changes as a result of 3'UTR length. CAT enzyme activity was measured from lysates of transiently transfected PF cells cultured under glucose-rich or deplete media, with units determined by comparison to standard curve. (Hatched bars-+Glc; solid bars –Glc

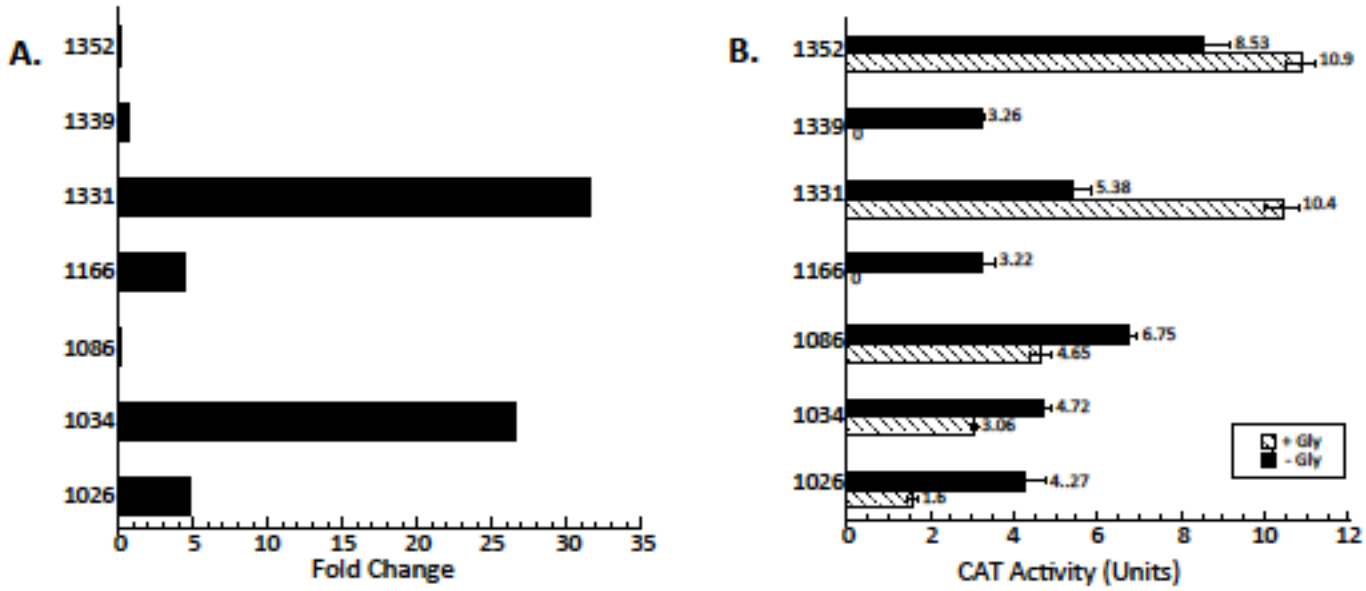

Figure 2.3 Growth in glycerol also alters steady state *TbHK1* transcript and expression in a 3'UTR-dependent fashion.

(A) Steady state transcript levels or (B) CAT activity from PF cells transiently transfected with reporter constructs followed by growth with (hatched bars) or without (solid bars) glycerol supplementation of SDM-80.

Impact of growth conditions on TbHK2 *expression and activity* – The single 353 nt 3'UTR found on the *TbHK2* transcript also influenced steady state abundance and expression in a nutrient-sensitive manner. Growth in the presence of glucose, but not glycerol, yielded a 3.6-fold increase in steady state transcript abundance. At the level of CAT expression, growth in either glucose or glycerol yielded similar expression levels, while growth in the absence of either carbon source led to a loss of detectable CAT activity.

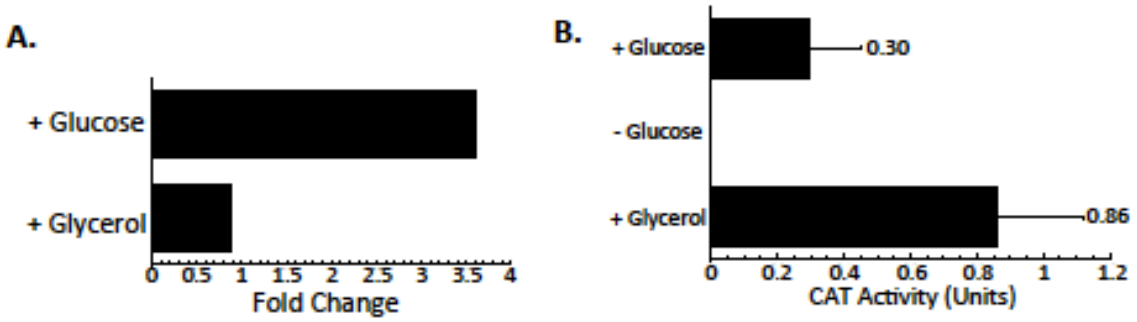

Figure 2.4. TbHK2 3'UTR yields differential expression for cells grown in the presence of glucose or glycerol.

(A) Steady state transcript levels or (B) CAT activity from PF cells transiently transfected with a reporter construct harboring the TbHK2 3'UTR followed by growth in the presence of glucose or glycerol. Steady state transcript levels are relative to transcript abundance in the absence of glucose or glycerol, with all levels normalized to actin.

TbHK enzyme activity is dependent on carbon source availability - HK activity in the African trypanosome is the result of multiple layers of regulation that include mechanisms that regulate steady-state transcript abundance and expression. Other mechanisms, including post-translational modification and oligomerization with TbHK2 (Chambers et al., 2008), are likely involved in regulation of TbHK activity, leading us to consider the impact of carbon source availability on cellular HK activity. Cells grown in media supplemented with glucose had the lowest TbHK activity per cell, while parasites grown in media lacking glucose or supplemented with glycerol had 2.3 and 3.3-fold higher TbHK activity, consistent with the general increase in CAT expression observed in cells grown under the two conditions (Fig. 2.4).

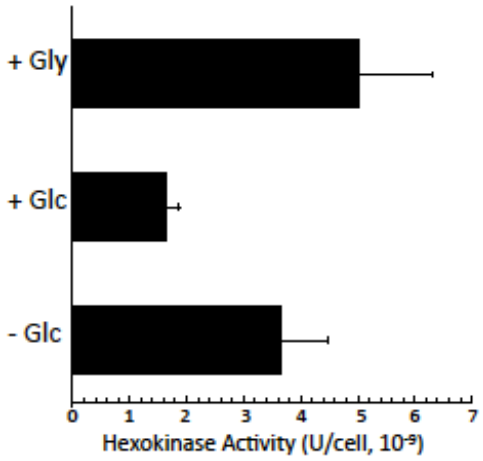

Figure 2.5 Growth conditions alter TbHK activity from PF lysates.

Trypanosomal lysates prepared by hypotonic lysis of cells grown in media containing the indicated carbon source for 24 hr were assayed for hexokinase activity as described in Materials and Methods.

DISCUSSION

Cellular mechanisms for responses to changes in nutrient availability are found in organisms as diverse as microbes and man. They are responsible for a fundamental process and include transcriptional, post-transcriptional, translational, and post-translational mechanisms but are unified in their importance for ensuring continued satisfaction of metabolic needs as environmental conditions change. Here, we have described how differential polyadenylation generates mRNA species that bear 3'UTRs that influence both steady-state transcript abundance and expression in response to environmental growth conditions.

Polyadenylation is critical for generation of the 3' ends of mRNAs in eukaryotes. Additionally, it provides a mechanism for influencing transcript maturation, particularly when intronic polyadenylation sites yield alternatively spliced message. Such a mechanism allows mammalian cells to generate isoforms of a given protein, and bioinformatic analysis suggests that ~50% of human genes have multiple polyadenylation signals, with ~20% having at least one intronic polyadenylation site (Gong et al., 2010; Tian et al., 2007; Tian et al., 2005).

While the *TbHK1* gene lacks introns, a similar mechanism may be at work here. This model is distinct from regulation of gene expression through control of pre-mRNA processing, which is not anticipated because the genes found in polycistrons in *T. brucei* are functionally unrelated and not organized into operon-like clusters (Berriman et al., 2005). Instead, pre-mRNA is matured as described, with the 5' addition of the SL to the 3' gene providing information for cleavage and polyadenylation of the 3' end of the 5' gene. Regulation, however, does occur at the choice of 3' cleavage sites in response to environmental cues. In eukaryotes, mRNA polyadenylation typically prevents message degradation, but in some cases can promote it, leading us to speculate that steady-state transcript abundance could be a reflection of

preferential degradation of some forms of *TbHK1* message – with maintenance of others – depending on the polyadenylation site (Table 1). This model is transcription-independent, which is key in the *T. brucei* system (because of the polycistronic transcription). Alternatively, (or additionally), maturation may not occur at all sites at equal frequency in response to environmental cues, leading to an increase in some species over others. In either case, alternative polyadenylation would likely yield mRNAs that bear different regulatory elements that then impact transcript stability and expression, depending on environmental cues.

	3'UTR Length (nt)	Transcript Abundance		CAT Expression		
		+Glc	+ Gly	-Glc	+Glc	+Gly
TbHK1	1026	+	+++	+++	+++	+
	1034	+++	++++	+++	+	+++
	1086	-	+	+++	+++	+++
	1166	+	+++	+++	+	-
	1331	+	++++	+++	+	+++
	1339	+++	+	+++	-	-
	1352	-	-	+++	-	++++
TbHK2	353	+++	+	-	+	+

Table 2.1. Summary of the impact of different TbHK UTR lengths on transcript abundance and CAT expression.

In agreement with previously published work (Siegel et al., 2010), the 1339 nt 3'UTR-bearing TbHK1 transcript was the most abundant message in media supplemented with glucose (Fig. 2.2A). Interestingly, protein expression was not detected from the same construct when cells were grown in the presence of glucose. This observation suggests that the transcript is synthesized and maintained but either lacks a cofactor required for protein synthesis or binds an inhibitor of protein expression under glucose-replete conditions. The two 3'UTRs with lengths most similar to the 1339 nt 3'UTR (1331 nt and 1352 nt) were not maintained at the transcript

level, suggesting that the stabilizing sequence may be removed during maturation of the shorter transcript (1331 nt) or masked by the longer 1352 nt 3'UTR.

The interesting difference in response to environmental nutrients at both the transcript and expression levels of transcripts bearing the 1339 nt and 1331 nt 3'UTR suggests that 3'UTR variants are generated to yield transcripts bearing distinct response elements. The eight nucleotides (aacacttt) that distinguish the 1339 nt 3'UTR from the 1331 nt species are indeed over-represented in the genome, suggesting a potential functional bias. With an average 3'UTR length of 604 nt, one would expect the 8-mer to populate the 5948 genes almost 55 times, but the sequence is found 111 times in the 3'UTR. This may reflect an artifact of a bias for A/T in the 3'UTR, but the sequence is found in the 3'UTRs of all five glycerol kinase (GK) genes (Tb09.211.3540, Tb09.211.3550, Tb09.211.3560, Tb09.211.3570, and Tb09.211.3590), suggesting some regulatory function in response to glycerol. The observation that the constructs bearing the 1339 nt 3'UTR are suppressed relative to the 1331 construct in the presence of glycerol suggests that the sequence is a negative regulator. Its presence in the 3'UTRs of the GK genes may reflect a regulatory mechanism to prevent catastrophic consumption of ATP by the enzyme in the presence of glycerol.

The 8nt difference between the 1339 nt and 1331 nt 3'UTR length also shared some sequence homology with the previously identified translation enhancer 16-mer element found in procyclin 3'UTRs (Hehl, 1994). Predictive m-fold structures particularly to the 3' end of the two different UTR's reveal that the 1339 nt 3'UTR contains a stem-loop structure at the end of the UTR, whereas the 1331 nt length does not form this structure (Figure 2.6). Interestingly, the 16-mer element identified in procyclin also forms a stem-loop structure and is not functional when

this RNA structure is lacking. Further characterization of this sequence is needed to fully determine the role that it plays in the regulation of *TbHK1* transcript level and expression.

A.

B.

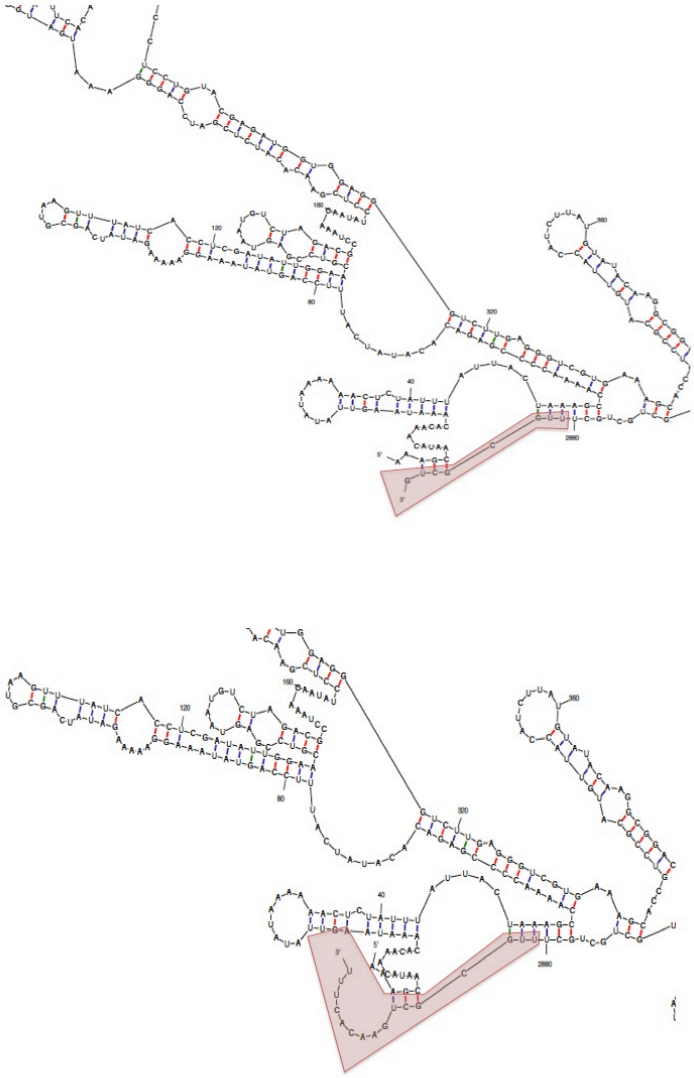

Figure 2.6. m-fold Structure of 1331nt and 1339 nt TbHK1 3'UTRs.

m-fold predicated structure of (A) 1331 nt (B) 1339 *TbHK1* 3'UTR. Homology shared with the procyclin 16-mer 3'UTR regulatory element (Hehl et al., 1994) is highlighted in red.

Overall, our study reveals that differential polyadenylation is not stochastic, but rather is involved in conferring regulation of gene expression of the glycolytic enzyme, TbHK1. The high number of genes with multiple polyadenylation sites from two recent RNA-seq efforts (Siegel et al., 2010; Kolev et al., 2010) suggests that the parasite likely employs this mechanism of regulation for many genes, in order to rapidly and efficiently adapt to gross and subtle environmental changes.

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CHAPTER 3

GLYCEROL-3-PHOSPHATE ALTERS *TRYPANOSOMA BRUCEI* HEXOKINASE ACTIVITY IN RESPONSE TO ENVIRONMENTAL CHANGE

Heidi C. Dodson, Meredith T. Morris, and James C. Morris

Department of Genetics and Biochemistry

Clemson University

Clemson, SC 29634

ABSTRACT:

The African trypanosome, *Trypanosoma brucei*, compartmentalizes some metabolic enzymes within peroxisome-like organelles, glycosomes. The amounts, activities, and types of glycosomal enzymes are modulated coincident with developmental and environmental changes. Pexophagy, fusion of glycosomes with acidic lysosomes, has been proposed to facilitate this glycosome remodeling. *In vitro*, the recombinant glycosomal enzyme *T. brucei* hexokinase 1 (rTbHK1) was inactivated under acidic conditions. Glycerol-3-phosphate (Gly3P), which is produced in the glycosome by glycerol kinase, mitigated acid inactivation of rTbHK1 and parasite lysate-derived TbHK activity. Glycerol-2-phosphate likewise prevented inactivation, while glycerol did not.

Gly3P influences rTbHK1 activity at pH 6.5 by preventing substrate and product inhibition by ATP and ADP, respectively. Gly3P protects rTbHK1 from quercetin (QCN) inhibition at pH 7.4 partially and completely at pH 6.5. However, Gly3P does not alter the

interaction of QCN with TbHK1, as the lone Trp (Trp177) was quenched under all conditions tested. These findings suggest potential novel mechanisms for the regulation of TbHK1, particularly given the changes in cellular concentrations of Gly3P and the acidification of glycosomes that can be induced under a variety of parasite growth conditions.

INTRODUCTION:

Regulation of metabolism is a central response to environmental changes that the parasitic protozoan *Trypanosoma brucei* encounters in its lifecycle. Metabolic pathways are adapted for nutrients available in either the tsetse fly vector or mammalian host. For example, procyclic form (PF) parasites residing in the tsetse fly metabolize both glucose and amino acids, while glycolysis is the sole ATP source for bloodstream form (BSF) trypanosomes.

Trypanosomes compartmentalize parts of the glycolytic pathway in an organelle called the glycosome. These organelles contain the first six or seven enzymes of glucose metabolism as well as enzymes involved in ether lipid biosynthesis and fatty acid oxidation. In addition, two enzymes involved in glycerol metabolism, glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol kinase (GK) are localized to this compartment (Opperdoes and Borst, 1977).

In the glycosome, glucose is initially converted to fructose-1,6-bisphosphate (FBP), a process that requires the consumption of ATP by both the trypanosome hexokinase (*Tb*HK) and phosphofructokinase (*Tb*PFK). These two enzymes are unusual in that they are insensitive to feedback inhibition by their products (Nwagwu and Opperdoes, 1982; Cronin and Tipton, 1985, 1987). One result of compartmentalization is that ATP production and consumption within the glycosome is balanced, with net ATP only synthesized in the cytosol. It has been suggested by modeling experiments that compartmentalization prevents the unproductive consumption of the net ATP (referred to as "turbo-explosion") by either the *Tb*HKs or *Tb*PFK (Haanstra et al., 2008). RNA interference (RNAi) studies of genes essential to glycosome biosynthesis support this observation, as glucose is toxic to PF *T. brucei* lacking glycosomes, presumably as a result of inappropriate consumption of cytosolic ATP (Furuya et al., 2002), while RNAi of *Tb*HK in the glycosome deficient cells is protective (Kessler and Parsons, 2005).

The "turbo-explosion" model suggests compartmentalization protects the cell from inappropriate kinase consumption of ATP. Additional layers of *Tb*HK regulation have recently been identified, including alteration of enzyme activity as a result of changes in the oligomerization state of the enzyme. Trypanosomes express two 98% identical *Tb*HKs (TbHK1 and TbHK2) that have different biochemical properties, including distinct sensitivity to pyrophosphate. Changes in oligomer composition can influence *in vitro* enzyme activity and sensitivity to pyrophosphate (Chambers et al., 2008). As further evidence of TbHK regulation mechanisms, TbHK1 can be inhibited by fatty acids that are found within the glycosome (Morris et al., 2006).

The glycosomal protein repertoire is altered in response to developmental and environmental changes. For example, as the parasite transits from the short stumpy form (which is found in the mammalian blood and are preadapted for life in the tsetse fly vector), glycosomes fuse with lysosomes (Herman et al., 2008). A process similar to pexophagy, which is the autophagy of peroxisomes in response to metabolic changes found in yeast, has been implicated in the turnover of glycosomal components. Here we demonstrate that TbHK1, which is sensitive to pH as a result of substrate and product inhibition**,** is protected by glycerol-3-phosphate (Gly3P), a metabolite that accumulates under some growth conditions. These observations suggest a novel means of regulation of an essential activity in the African trypanosome.

MATERIALS AND METHODS

Reagents - ATP and ADP were purchased from Calbiochem (San Diego, CA). To confirm purity (reported by the manufacturer to be >95%), both compounds were resolved by HPLC (Waters, Milford, MA) using an isocratic buffering system (100 mM KH_2PO_4 , pH 6.5, 1.2% methanol) on a Symmetry C18 (5µm, 4.6 x 150 mm) column at a flow rate of 1.0 ml/min. Compounds were detected at 259 nm on a dual UV detector and analyzed by Breeze 3.2V software. Antisera to *T. brucei* glycerol kinase (TbGK) (1:100,000), *T. brucei* hexokinase (TbHK, which detects both TbHK1 and TbHK2) (1:100,000), *T. brucei* triosephosphate isomerase (TbTIM) (1:3,500), and *T. brucei* enolase (TbENO) (1:10,000) were the generous gift of Dr. Paul Michels (Laboratory of Biochemistry, Université catholique de Louvain). Antiserum to T. brucei PEX11 (TbPEX11) (1:4,000) was the gift of Dr. Christine Clayton (Center for Molecular Biology Heidelberg, University of Heidelberg). Quercetin (3,3',4',5,7-pentahydroxyflavone) was purchased from Spectrum Chemical Manufacturing Corporation (Gardena, CA).

Cell Culture and Transformation - Both procyclic and bloodstream form (PF and BSF) *Trypanosoma brucei brucei* strain 427 (29-13 and 90-13, respectively) were used in the cellular studies. PF parasites were grown in SDM 79 (Wirtz et al., 1999; Wang et al., 2000) to a density of $1x10^7$ cells/ml, collected by centrifugation (10 min, 3000 x g), and stored at -20°C until use. To lyse cell pellets, the parasites were thawed, washed, and resuspended in ddH_20 to a final concentration of $1x10^8$ cells/ml. Lysis buffer (0.1% Triton X-100, 50 mM TEA, pH 7.4, 1 mM PMSF, 20mg/mL leupeptin, 100mg/mL TLCK) was added to the mixture to minimize protein

degradation upon lysis. BSF parasites were treated similarly, with cell pellets frozen initially at a concentration of $1x10^6$ cells/ml.

Recombinant Enzyme and Assay Conditions- For purification of rTbHK1, a previously described protocol (Morris et al., 2006) was modified to increase yield. Briefly, a starter culture of *E. coli* M15(pREP) harboring pQE30 (Qiagen, Valencia, CA) with TbHK1 cloned in frame of a 6-His tagging sequence was grown in ECPM1 (Wigelsworth et al., 2004). The starter culture was used to inoculate a 5 L culture in a bioreactor (Biostat B, B. Braun Biotech International, Allentown, PA) at 37^{o}C supplemented with O_2. The culture was induced with 0.8 mM IPTG when the culture reached an OD_{600} between 3-5, and allowed to grow for 16 hours without O_2 supplementation. The cell pellet was collected by centrifugation (5000 x g, 20 min, 4^{o}C) and resuspended in His buffer (50 mM $NaPO_4$, pH 8.1, 5 mM glucose, 150 mM NaCl, 0.1% Tween). Cells were passed through a cell disruptor (Constant Cell Disruption Systems, Sanford, NC) for lysis. After clearing the lysate by centrifugation, the supernatant was run on a 50 ml ProBind column (Invitrogen, Eugene, OR) at a rate of 5 ml/min on a FPLC (GE Lifesciences, Piscataway, NJ) with a step gradient of 5 to 250 mM imidazole in His buffer. Fractions were screened using HK activity assays and Western blotting and those containing rTbHK1 were pooled and concentrated. Further purification by ion-exchange chromatography was performed using a HiTrap SP HP column (GE Lifesciences, Piscataway, NJ) with a gradient elution. At the end of the purification, the protein was determined to be ~99% pure based on SDS-PAGE.

HK assays were performed as described in triplicate using a coupled reaction to measure enzyme activity (Morris et al., 2006; Misset and Opperdoes, 1984). In short, the coupled assay uses glucose-6-phosphate dehydrogenase (G6PD) to convert glucose-6-phosphate (G6-P)

generated by HK to 6-phosphogluconate with coincident reduction of NADP to NADPH, which is monitored spectrophotometrically at 340 nm. Reagent final concentrations in the 200 µl reactions were (unless otherwise noted): 50mM triethanolamine (TEA)/ 3-(N-morpholino) propanesulfonic acid (MOPS)/ piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES), 3.3mM $MgCl_2$, 20mM glucose, 5.25mM ATP, 0.75 mM NADP, and 0.1 units of G6PDH. For protection assays, Gly3P (20 mM) was added with the other assay components. Kinetic analyses were performed using KaleidaGraph 4.1 (Synergy Software, Reading, PA) with the Michaelis-Menten curve fit algorithm.

Enzyme activity in parasite lysates - PF or BSF parasites were lysed as described above. Cell equivalents (1×10^5 or 4×10^5 for PF and BSF, respectively) were mixed with all HK assay components and the HK reaction monitored spectrophotometrically.

Tryptophan Quenching Assay of TbHK1 –Quercetin (QCN) (varying concentrations) and Gly3P (20mM) were added individually and in combination to a solution (3 mL) of 0.1M MOPS, pH 6.5. A scanning spectrofluorometer (QM-Y, Photon Technology International, Birmingham, NJ) was used to monitor emission from 300-550 nm after excitation of the lone Trp on TbHK1 (W177) at 280 nm (Dodson, 2010). Once background emission was acquired, ~1 ug of TbHK1 was added to the cuvette, mixed by pipetting and a second emission scan was performed. Using Photon Technology International (PTI) software, the area under the emission curve from 370-380nm was integrated. The values were converted to Trp emission lost and plotted versus concentration of QCN using KaleidaGraph 4.1 (Synergy Software, Reading, PA) to determine IC_{50} values.

RESULTS

Starvation triggers altered abundance of some glycosome-resident proteins. PF trypanosomes can adapt their metabolism to conditions of low glucose, presumably by up-regulation of amino acid metabolism (ter Kuile, 1997; McCann et al., 2008). Because of this dynamic metabolism, PF parasites can grow normally in the near absence of glucose. Complete denial of nutrients by incubation in phosphate-buffered saline, however, has been shown previously to trigger association of glycosomes with the lysosomal compartment in PF cells, a process similar to pexophagy in yeast (Herman et al., 2008).

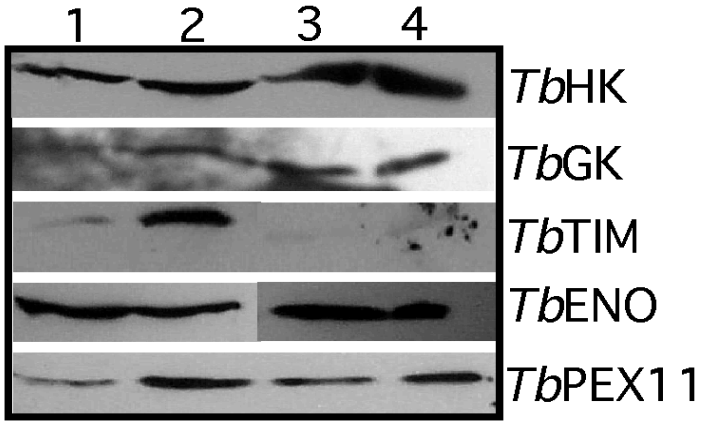

Figure 3.1. Incubation of PF parasites in PBS leads to altered abundance of glycosome-resident proteins.

Whole cell lysate from parasites maintained in SDM 79 ($5x10^6$ or $1x10^7$ cells, lane 1 and 2, respectively) or incubated in PBS for 15 min ($5x10^6$ or $1x10^7$ cells, lane 3 and 4) were probed with antisera raised against glycosomal residents (*Tb*GK, *Tb*HK, *Tb*TIM), a glycosomal membrane protein (*Tb*PEX11) (Lorenz et al., 1998) and a cytosolic protein (*Tb*ENO).

To assess the consequence of association of glycosomes with acidic compartments, parasites were grown under standard conditions or incubated in PBS, a process known to trigger trypanosomal autophagy (Herman et al., 2008) and cell lysates probed with antisera to glycosomal resident proteins and cytosolic proteins. Incubation in PBS led to a modest decrease in *Tb*HK signal (likely a mixture of TbHK1 and TbHK2, as the antisera does not discriminate) (Figure 3.1, lanes 3 and 4). *Tb*GK abundance is not depleted, while *Tb*TIM levels are markedly reduced. The impact of incubation in PBS is not systemic, as the cytosolic-resident glycolytic enzyme *Tb*ENO and the glycosomal membrane protein *Tb*PEX11 are not impacted. These findings are similar to those reported in earlier studies on autophagy (pexophagy) of glycosomes that occurs after brief incubation of cells in PBS or during differentiation (Herman et al., 2008).

TbHK1 is pH sensitive, but Gly3P prevents inactivation. To explore the potential impact of fusion of the glycosome with the acidic (pH 4.8) lysosome (McCann et al., 2008) on a glycosome-resident protein *in vitro*, purified recombinant TbHK1 was assayed under different pH conditions. The recombinant enzyme, normally assayed at pH 7.4, is sensitive to acidic conditions, with ~ 50% reduction in activity at pH 7.0 and nearly complete loss of activity at pH 6.5 (Fig. 3.2A).

Glycosomes are dynamic organelles that are subject to changes in pH and enzyme and metabolite content, depending on both the environment in which the cell resides and the life cycle stage. For example, PF parasites grown in amino acid-based media rapidly supplemented with glycerol quickly accumulate Gly3P from undetectable levels to ~ 15 mM within an hour (Haanstra et al., 2008). This increase is attributed to the activity of the sole cellular glycerol kinase (GK), a glycosome-resident protein (Haanstra et al., 2008). The dynamic nature of

glycosome contents led us to consider the impact of Gly3P on pH-mediated inactivation of TbHKs.

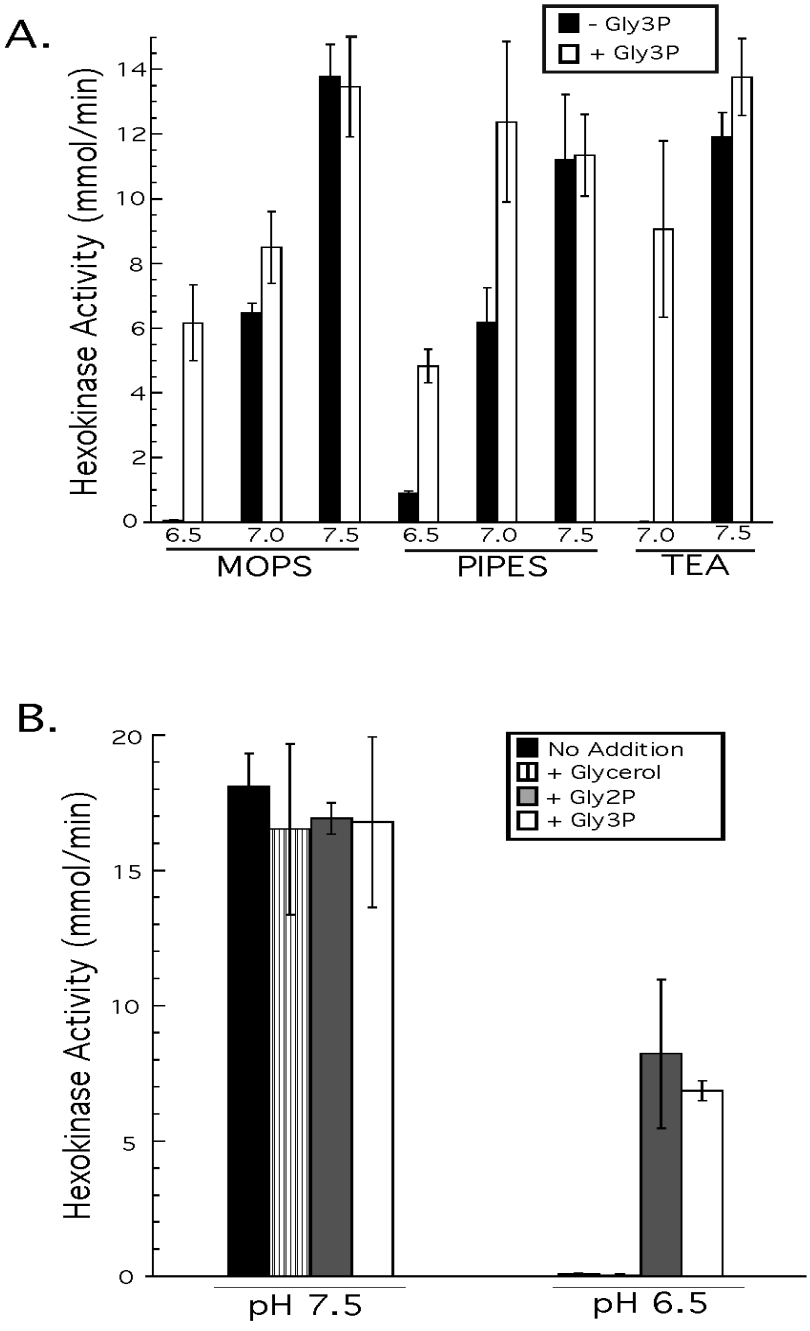

Figure 3.2. TbHK1 inhibition under acidic conditions can be prevented by inclusion of Gly3P in the assay.

(A) pH profile of TbHK1 (150 ng) activity in the presence (white) or absence (black) of Gly3P. Three different Good's Buffers with buffering capacity in the range of pH tested were used for the assay: MOPS, PIPES and TEA. (B) TbHK1 activity in MOPS buffer at pH 7.4 or 6.5 with or without Gly3P, Gly2P, or glycerol (Gly) (20 mM each).

Performing TbHK1 assays under acidic conditions in the presence of Gly3P modulated pH inactivation of the enzyme (Fig. 3.2A). At pH 6.5, more than 50% of the TbHK1 activity observed at pH 7.4 was maintained when Gly3P was included. Notably, detectable enzyme activity was maintained at pH 4.0 (the lowest pH tested) in the presence of Gly3P (Figure 3.3).

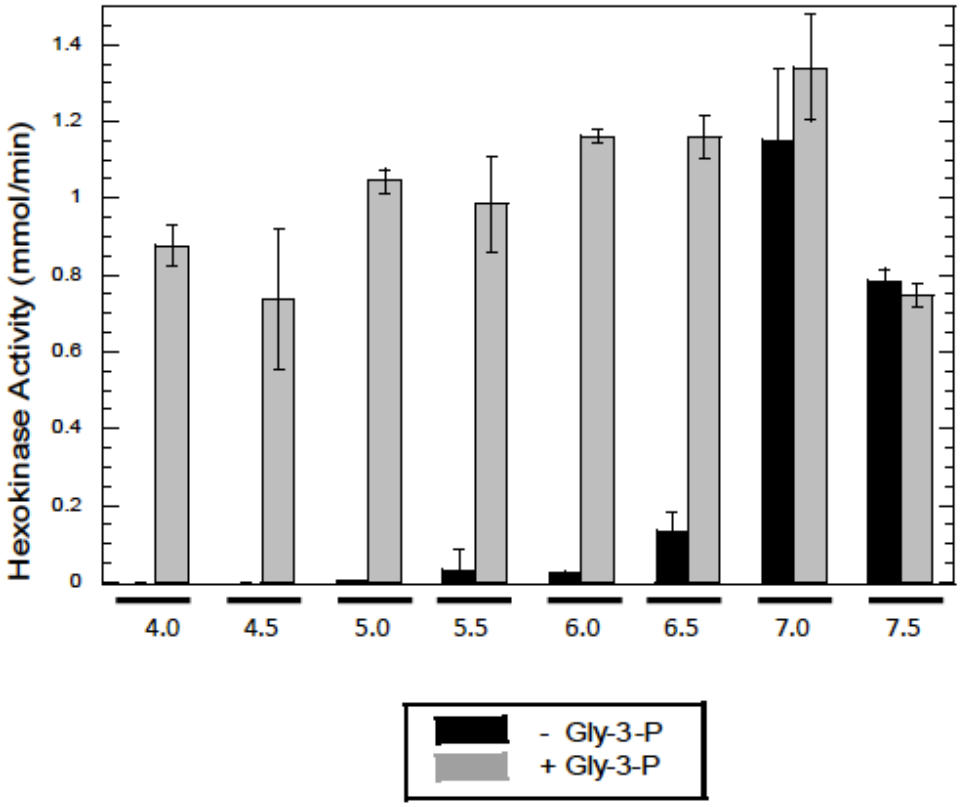

Figure 3.3 pH profile of rTbHK1 activity +/- Gly3P

Hexokinase assays were conducted in reaction mixture using 50 mM TEA buffer from pH 4.0 to 8.0 with ATP as a phosphoryl donor.

The pH of the reaction mixture remained unchanged after Gly3P addition, ruling out the possibility of Gly3P buffering the reaction. In parallel experiments lacking Gly3P, the enzyme was inhibited at pH 7.0 (Fig. 3.2A). Gly2P also prevented inactivation while glycerol did not (Fig. 3.2B). Gly3P has no impact on TbHK1 oligomerization (a previously characterized mechanism for regulation of activity (Chambers et al., 2008)) indicating that the maintenance of enzyme activity that we observed is not related to a change in the nature of the hexamer (Fig. 3.4). While Gly3P may be serving as a molecular crowding agent that abrogates the impact of pH or temperature, substituting glycerol or PEG did not prevent inactivation, suggesting that if the observed results were due to crowding, it was a specific response to glycerophosphate compounds.

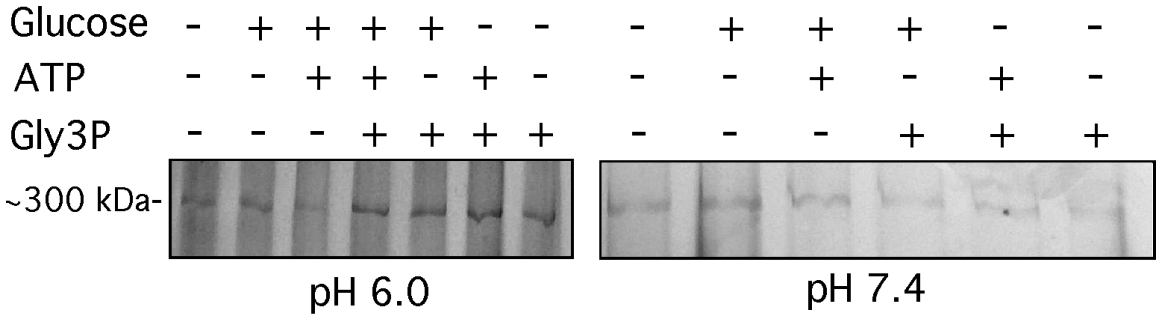

Figure 3.4. Resolution of rTbHK1 oligomers at pH 7.4 with various metabolites present.

rTbHK1 was incubated for 30 minutes at pH 7.4 in the presence of glucose (Lanes 2, 9) glucose and ATP (Lanes 3, 10), glucose, ATP and Gly3P (Lane 4), glucose and Gly3P (Lanes 5, 11), ATP and Gly3P (Lanes 6,12) or Gly3P alone (Lane 7,13) prior to resolution on a 4% native gel (as described in Chambers et al., 2008).

To ensure that the protection of the recombinant enzyme was not an artifact of the coupled enzyme assay, G6PDH was incubated at pH 6.0 with G6-P as substrate, thereby foregoing the need for HK to produce G6-P from ATP and glucose. G6PDH activity was unaffected by the acidic pH, readily converting G6-P to 6-phosphogluconate at the acidic pHs tested here, in the presence or absence of ATP and glucose (Fig. 3.5). Therefore our observations are not a result of altering the G6PDH coupled enzyme activity.

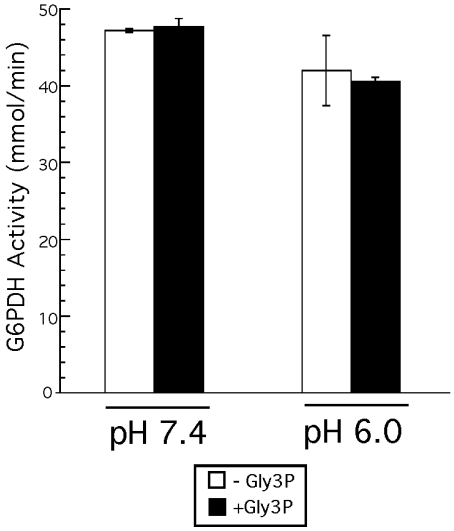

Figure 3.5. G6PDH activity at pH 7.4 and pH 6.0 with or without Gly3P.

A reaction mix of G6PDH (0.1 units/reaction), glucose-6-phosphate (20mM), and NADP (0.75mM) buffered to pH 7.4 (left) or 6.0 (right) was supplemented with or without Gly3P (20mM) and G6PDH activity measured spectrophotometrically at 340 nm.

Gly3P and Gly2P, but not glycerol, also maintained authentic TbHK activity from both procyclic form and bloodstream form cell lysate at low pH (Figs. 3.6A and 3.6B). These findings again suggest that the glycerol moiety is not merely serving as a protective agent but rather that the phosphate is important in maintaining activity.

ATP and ADP are inhibitory under acidic conditions. The observation that Gly3P or Gly2P altered rTbHK1 activity led us to consider whether the change in pH was somehow altering the interaction of the protein with other phosphoryl-bearing compounds, including the substrate ATP. At pH 7.4, a broad range of ATP concentrations support TbHK1 activity with an inhibitory consequence on activity only at very low (0.1 mM) and very high (15 mM) concentrations. At pH 6.5, however, the enzyme is more sensitive to the substrate, with optimal activity at ~1 mM (Fig. 3.7A) and inhibition at higher concentrations. The affinity for ATP does not change dramatically at pH 6.5 (apparent Km of 0.22 ± 0.17 mM, compared to 0.28 mM at pH 7.4 (9)). The addition of Gly3P only slightly increases the apparent affinity to 0.16 ± 0.07 mM at pH 6.5.

Supplementing the low concentration of ATP (1 mM) needed to fuel the TbHK1 reaction at pH 6.5 with ADP inhibited the enzyme (Fig. 3.7B). ADP is a mixed inhibitor of TbHK1 (Fig. 3.7C), with an IC_{50} of 4.2 mM. The impact of ADP on the assay at pH 7.4 was reduced, yielding an IC_{50} of ~ 10 mM. Similar experiments using AMP in lieu of ADP did not inhibit the enzyme. Additionally, introduction of additional $MgCl_2$ into the reaction did not reverse inhibition, indicating that the inhibition was not a result of the nucleotide depleting Mg^{2+} required for enzyme function.

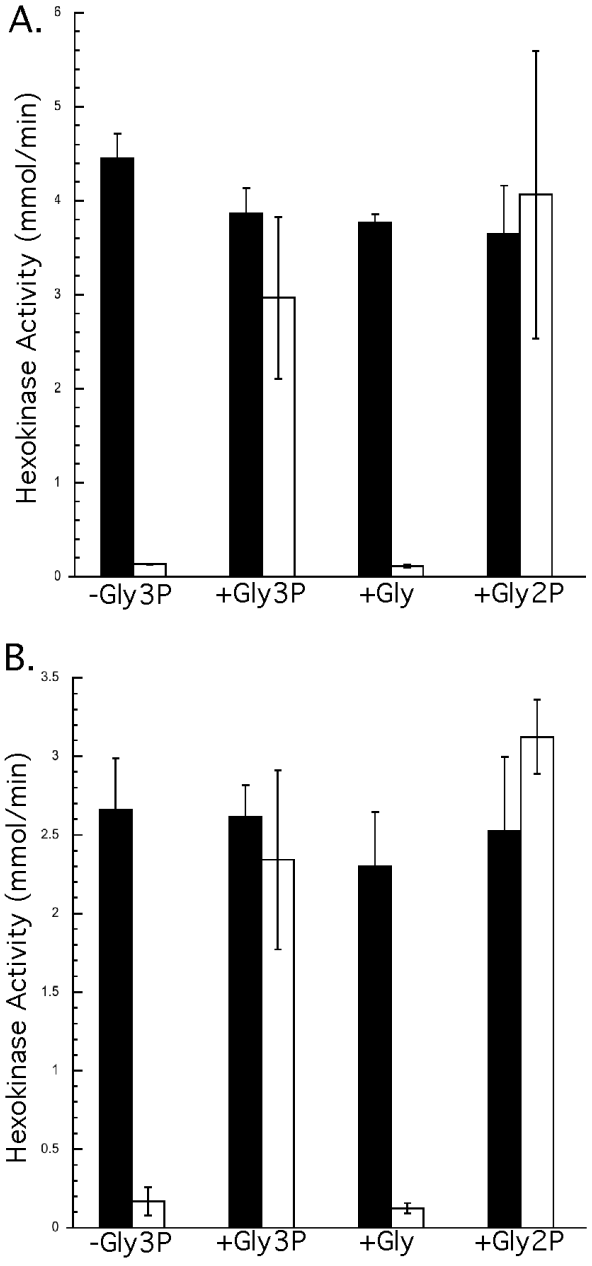

Figure 3.6. Hexokinase Activity in Cell Lysate with various glycerol moieties.

BSF lysate (4 x 10^5 cell equivalents (A)) or PF lysate (1 x 10^5 cell equivalents, (B)) were assayed for HK activity at pH 7.4 (black) and pH 6.5 (white) with or without the addition of Gly3P, Gly2P or Gly (all at 20 mM).

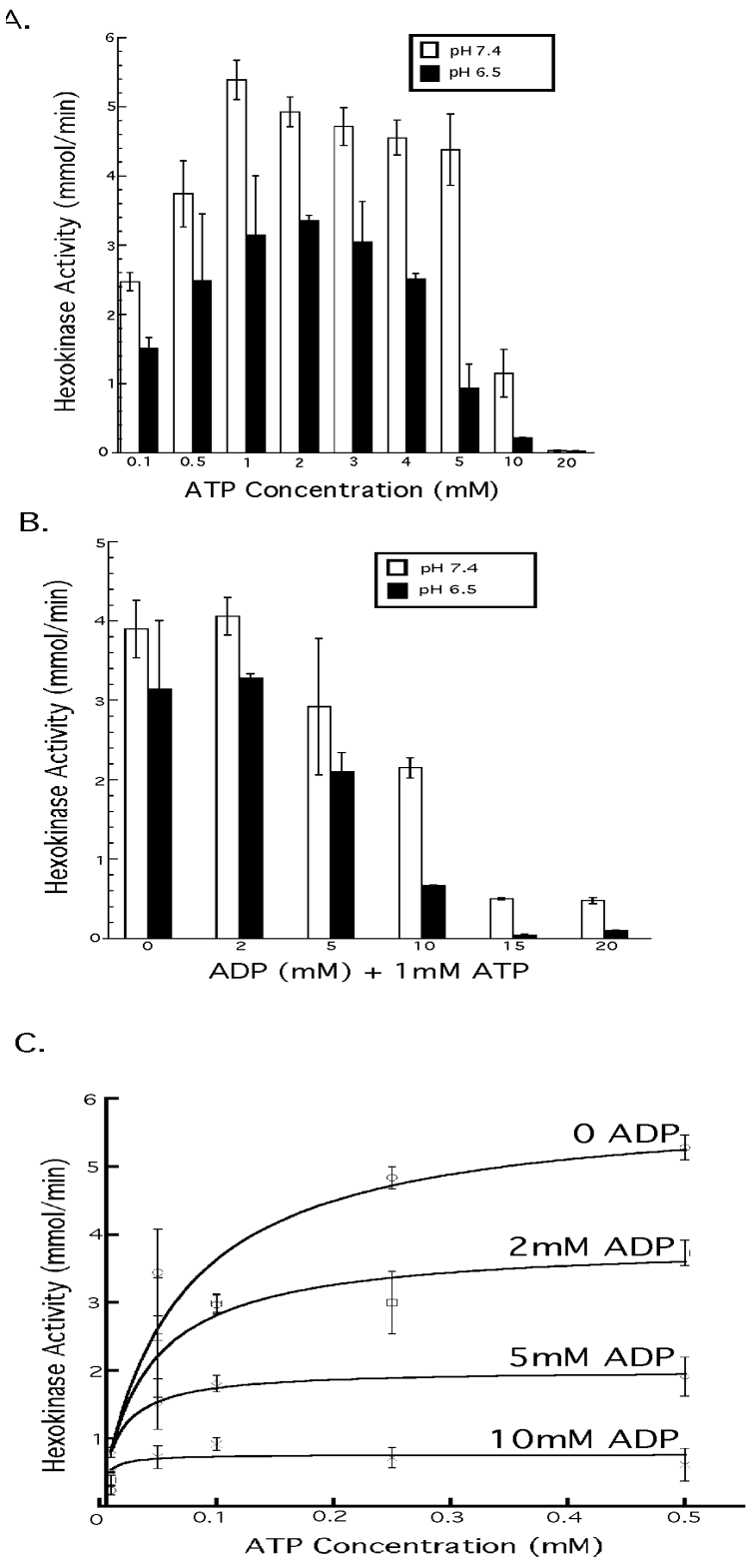

Figure 3.7. Substrate and product inhibit TbHK1 under acidic conditions.

(A) TbHK1 (150 ng) activity was assayed under acidic conditions (pH 6.5) including ATP from 0-20 mM in the assay. (B) TbHK1 activity assessed in the presence of 1 mM ATP (competent for activity) supplemented with ADP (0-20 mM) at pH 6.5. (C) ADP is a mixed inhibitor of TbHK1 at pH 6.5. Assays, performed as described in the Materials and Methods, were plotted using KaleidaGraph 4.1 (Synergy Software, Reading, PA) with the Michaelis-Menten curve fit algorithm.

Quercetin inhibition, but not binding, is relieved by Gly3P at pH 6.5. To further characterize the interaction of Gly3P with TbHK1, we employed a fluorescent probe, quercetin (QCN), which inhibits TbHK1 with an IC_{50} of 4.1 ± 0.8 μM at pH 7.4 (Dodson et al., 2010). Additionally, QCN quenches fluorescence from the lone Trp residue (Trp177) that lies near the enzyme hinge adjacent to the catalytic base, Asp214 (Dodson et al., 2010). At a concentration of QCN near the IC_{50} (10 mM), Gly3P prevents inhibition at pH 7.4, but fails to prevent inactivation by higher QCN concentrations (Fig. 3.8A). Notably, at pH 6.5, QCN inhibition is abolished in the presence of Gly3P.

To explore the consequences of Gly3P on QCN binding, TbHK1 was incubated with increasing concentrations of QCN in the presence or absence of Gly3P and the consequences on Trp177 emission assessed. Under standard conditions (pH 7.4), QCN quenched 50% of Trp177 emission at 35 μM (Dodson et al., 2010). At pH 6.5, emission was similarly quenched by QCN, independently of the presence of Gly3P (yielding 50% quenching at 43 and 50 μM QCN, with or without Gly3P, respectively) (Fig. 3.8B).

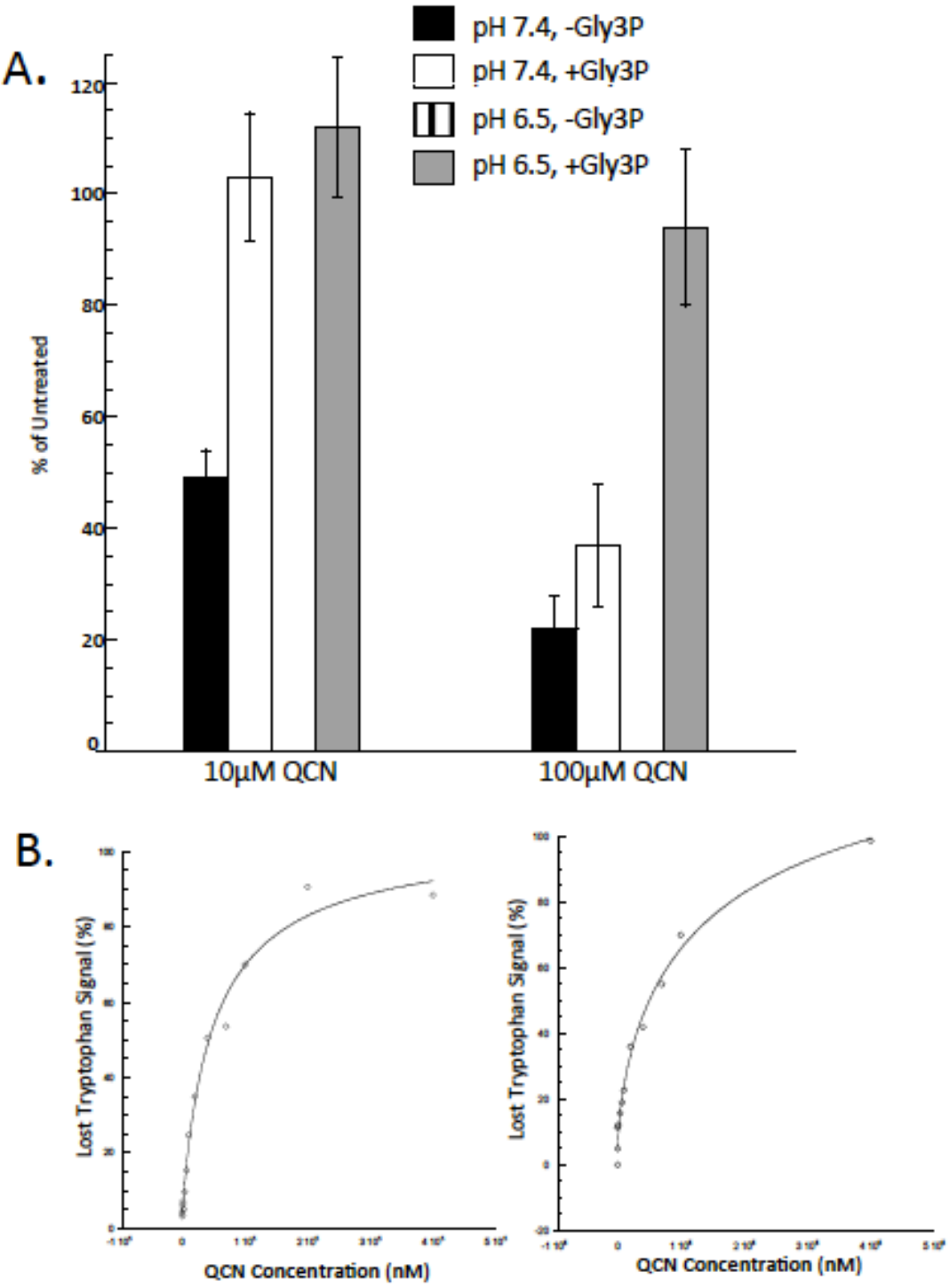

Figure 3.8. QCN inhibition is relieved in the presence of Gly3P at pH 6.5.

(A) TbHK activity in the presence or absence of Gly3P with varying concentrations of QCN (10 or 100 μM). (Please note that at pH 6.5 in the absence of Gly3P, activity is not detected, hence the lined bar is not visible.) (B) Trp177 emission is quenched by QCN at pH 6.5 in the absence (left) or presence (right) of Gly3P. Increasing concentrations of QCN were incubated with rTbHK1 in a 100 mM MOPS, pH 6.5 solution. Trp177 was excited at 280 nm followed by an emission scan from 300-550nm. The percentage of Trp emission lost upon addition of QCN was calculated from the emission peak between 370-380 nm.

DISCUSSION

T. brucei alternates between a tsetse fly vector and mammalian host, utilizing changes in environmental nutrient availability as part of a complex pathway to regulate development. These changes include activation of "cryptic" mitochondrion in the BSF to a metabolically active organelle in the PF parasite. Additionally, glycosome contents are altered in a developmentally coordinated fashion. For example, BSF parasites metabolize glucose to 3-phosphoglycerate (3-PGA) within the glycosome, followed by further processing in the cytosol to ultimately yield pyruvate. PF parasites, however, process glucose to 1,3-bisphosphoglycerate in the glycosome, indicating that the parasite utilizes stage-specific differences in glycosomal content to regulate metabolic pathways.

Changes in glycosome content occur through glycosomal autophagy (Herman et al., 2008). During differentiation, glycosomes co-localize with lysosomes, which occurs particularly quickly (and involves a high percentage of glycosomes) during the transition from short-stumpy form parasites (the mammalian blood form pre-adapted for life in the insect) to PF parasites (Herman et al., 2008). As an example of the impact of this process, the levels of the glycosome-resident protein aldolase are reduced (compared to levels in BSF) in short stumpy forms, and then increased again in the PF form, only to slowly decrease thereafter. This process resembles

pexophagy, the mechanism for peroxisome turn-over in methylotrophic yeast that occurs when nutritional conditions change (Farre and Subramani, 2004).

Here we have found that a change in protein environment (a change in pH) impacts the response of TbHK1 to both substrate (ATP) and product (ADP), heretofore unrecognized as regulators of enzyme activity (Fig. 3.9). Product inhibition by ADP is not competitive with substrate, indicating that a secondary nucleotide binding site has been exposed in response to the acidic conditions of the assay. The change in protein structure is subtle, as the enzyme functions at reduced ATP concentrations. Under the more acidic pH conditions, QCN quenches Trp177 fluorescence, indicating that it interacts with the protein. However, the flavonol fails to inhibit the enzyme, suggesting that the subtle change in structure has moved the QCN binding site sufficiently far from the active site to allow activity (Fig. 3.9).

We expect total TbHK activity to differ between BSF and PF parasites as BSF parasites rely exclusively on glycolysis for ATP production. Reflecting metabolic differences in the life stages, glycolytic proteins make up ~90% of the protein content in BSF glycosomes, while in PF glycosomes, only ~40-50% of the protein content is glycolytic proteins (Michels and Opperdoes, 1991; Moyersoen et al., 2004; Michels et al., 2006). Nevertheless, TbHKs are important to both life stages, as TbHK1 and TbHK2 have been shown to be essential to BSF parasites (Chambers, 2008 and Albert, 2005), while TbHK2$^{-/-}$ PF parasites are viable but the knockout has a pleiotropic impact on the cells. Viable TbHK1$^{-/-}$ PF cells have not yet been isolated despite repeated attempts under different growth conditions (M.T. Morris, unpublished observation), suggesting TbHK1 is also essential to PF cells.

The importance of the interaction of Gly3P with *Tb*HKs, and the subsequent regulation of enzyme activity, can be inferred from parasites that lack glycosomes due to genetic manipulation

of the *PEX14* gene, which is involved in peroxisomal and glycosomal protein import. As a result of the *PEX14* deficiency, glycosome biogenesis is blocked, yielding parasites that grow normally in the absence of glucose. However, addition of glucose to cultures of *PEX14*-deficient parasites is toxic (Furuya et al., 2002). Glucose toxicity has been proposed to result from the lack of appropriate compartmentalization of *Tb*HKs and PFK, leading to uncontrolled consumption of ATP (Furuya et al., 2002). Interestingly, RNAi of *Tb*GK was found to rescue the parasites from glucose sensitivity in the *PEX14*-deficient background (Haanstra et al., 2008). This observation suggests that the absence of *Tb*GK leads to a reduction in cellular Gly3P that therefore allows pH inactivation of *Tb*HK activity. Without sufficient Gly3P to maintain *Tb*HK activity, the cell is protected from the lethal "runaway" activity of the enzyme.

FOOTNOTES

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The abbreviations used are: BSF, bloodstream form; G6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; Gly3P, glycerol-3-phosphate; GK, glycerol kinase; HK, hexokinase; PF, procyclic form; rTbHK1, recombinant *Trypanosoma brucei* hexokinase 1; TbHK, *T. brucei* hexokinase.

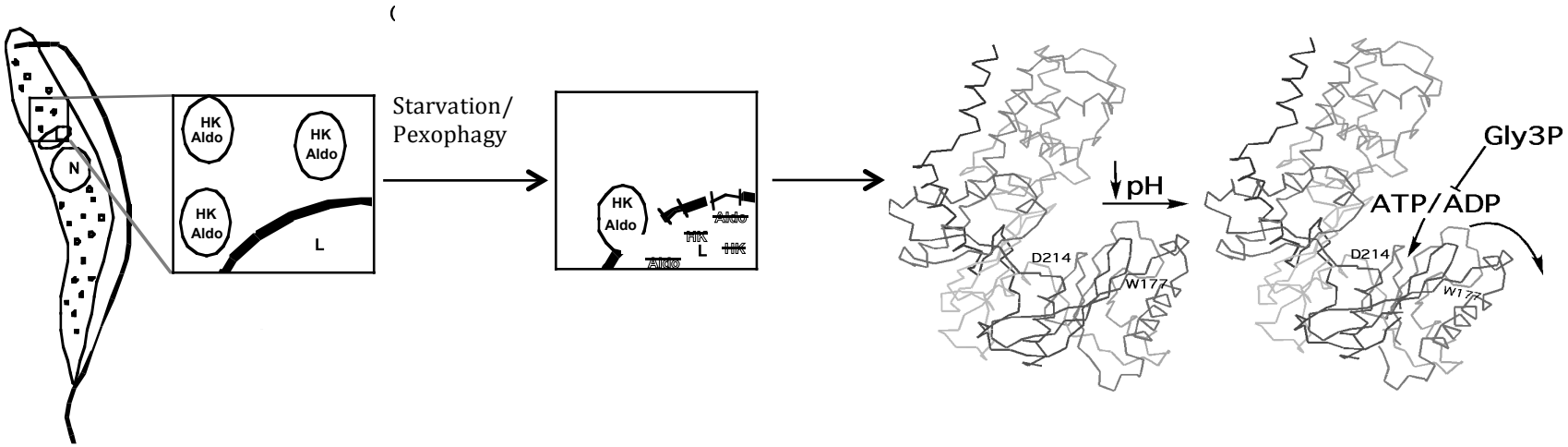

Figure 3.9. Model illustrating the potential consequences of pH and Gly3P on TbHK1 activity.

The active site catalytic base, Asp214, and the only Trp, Trp177, are indicated on TbHK1 modeled to the crystal structure of *S. cerevisiae* hexokinase PII (Kuser, 2000). This model lacks the unstructured N-terminal peroxisomal targeting sequence (PTS2). ATP/ADP binding at a non-catalytic site is indicated.

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CHAPTER 4

QUERCETIN, A FLUORESCENT BIOFLAVONOID, INHIBITS *TRYPANOSOMA BRUCEI* HEXOKINASE 1

Heidi C. Dodson, Todd L. Lyda, Jeremy W. Chambers, Meredith T. Morris, Kenneth A. Christensen, and James C. Morris

Department of Genetics and Biochemistry

Clemson University

Clemson, SC 29634

ABSTRACT

Hexokinases from the African trypanosome, *Trypanosoma brucei*, are attractive targets for the development of anti-parasitic drugs, in part because the parasite utilizes glycolysis exclusively for ATP production during the mammalian infection. Here, we have demonstrated that the bioflavonoid quercetin (QCN), a known trypanocide, is a mixed inhibitor of *Trypanosoma brucei* hexokinase 1 (TbHK1) (IC_{50} = 4.1 ± 0.8 μM). Spectroscopic analysis of QCN binding to TbHK1, taking advantage of the intrinsically fluorescent single tryptophan (Trp177) in TbHK1, revealed that QCN quenches emission of Trp177, which is located near the hinge region of the enzyme. ATP similarly quenched Trp177 emission, while glucose had no impact on fluorescence.

Supporting the possibility that QCN toxicity is a consequence of inhibition of the essential hexokinase, in live parasites QCN fluorescence localizes to glycosomes, the

subcellular home of TbHK1. Additionally, RNAi-mediated silencing of TbHK1 expression expedited QCN induced death, while over-expressing TbHK1 protected trypanosomes from the compound. In summary, these observations support the suggestion that QCN toxicity is in part attributable to inhibition of the essential TbHK1.

INTRODUCTION

Trypanosoma brucei is the causative agent of human African trypanosomiasis and nagana, a wasting disease, in livestock. The World Health Organization classifies *T. brucei* as a re-emerging/uncontrollable human pathogen, partly due to a lack of a vaccine and suitable treatments for the disease. Current therapeutics for human African trypanosomiasis (HAT) may have serious side effects, including blindness and death (Barrett et al., 2003).

T. brucei relies exclusively on glycolysis for ATP generation in the mammalian bloodstream. Hexokinases (HK) catalyze the first step in glycolysis facilitating the transfer of the γ-phosphoryl group of ATP to the C6 of glucose. The parasite expresses two HKs, TbHK1 and TbHK2, with proteomic studies revealing that both are found in the mammalian bloodstream (BSF) and insect (PF) forms of the parasites (Colasante, et al., 2006). Both the proteins reside in an unusual organelle called the glycosome that houses the majority of the enzymes that participate in glycolysis.

TbHK1 and TbHK2 are 98% identical at the amino acid level (Morris, et al., 2006). RNA interference (RNAi) has been used to demonstrate that both enzymes are essential to the BSF parasites, as silencing of either *TbHK1* or *TbHK2* results in the loss of HK activity and cell death (Albert et al., 2005; Chambers et al., 2008). In addition to this genetic evidence validating TbHKs as potential therapeutic targets, we have found that chemicals that inhibit HKs from other systems also inhibit TbHKs and are toxic to the trypanosome. For example, the anticancer drug lonidamine (LND), which functions in part by inhibiting human HK (Floridi and Lehninger, 1983; Paggi et al., 1988), inhibits

both recombinant TbHK1 and HKs from parasite lysate. Additionally, LND is toxic to BSF and PF parasites (Chambers et al., 2008), likely as a result (at least in part) of inhibition of TbHKs. Supporting this, parasites were partially protected from LND-induced cell death by ectopic over-expression of TbHK1.

Quercetin (3,5,7,3',4' pentahydroxyflavone, QCN) is an abundant naturally occurring flavanol found in plants such as apples, onions, and capers. QCN and related flavanols are of interest as potential anti-cancer therapies, because they inhibit the growth of several types of cancer cell lines (Molnar et al., 1981; Suolinna et al., 1975). Potential *in vivo* QCN targets include a number of enzymes that are inhibited *in vitro,* ranging from the Src protein kinase ($pp60^{v\text{-}src}$) to HKs (Graziani, 1977; Graziani, et al., 1983).

Biophysically, QCN has several unusual fluorescence properties, including intramolecular excited-state proton transfer and dual fluorescence behavior that have been exploited in the use of flavanols as environmental probes (Guharay et al., 2001). Additionally, QCN binding to bovine serum albumin has been studied using these spectral properties (Sengupta and Sengupta, 2002).

Here, we have characterized the impact of QCN on recombinant TbHK1 and transgenic parasites. Our work builds on the observation that QCN is toxic to trypanosomes (Mamani-Matsuda et al., 2004), revealing that TbHK1 may be a molecular target of the flavanoid. We have found that over-expression of TbHK1 provides protection from QCN, while RNAi depletion of TbHKs expedites parasite death. Additional spectroscopic investigations taking advantage of the intrinsic fluorescence of

QCN suggest that QCN toxicity may be due in part to binding near the TbHK1 active site, causing enzyme inactivation.

MATERIALS AND METHODS

Reagents

QCN (3,3',4',5,7-pentahydroxyflavone) was purchased from Spectrum Chemical Manufacturing Corporation (Gardena, CA).

Assays of recombinant and lysate-derived TbHK

Recombinant TbHK1 was expressed and purified as described previously (Morris et al., 2006). Parasite lysates were prepared by hypotonic lysis of 1 x 10^7 cells in the presence of 1 mM PMSF, 20 μg/ml leupeptin, and 100 μg/ml TLCK. The mixture was added to lysis buffer (for a final concentration of 0.1 M triethanolamine (TEA) pH 7.4 and 0.1% Triton X-100) and lysates used in HK assays.

HK assays were performed in triplicate using a coupled reaction. Briefly, assays used glucose 6-phosphate dehydrogenase (1 unit/assay, EMD Biosciences, Inc, Sand Diego, CA) as a coupling enzyme to reduce NAD^+ to NADH during the oxidation of glucose-6-P to 6-phosphogluconic acid (Morris, et al., 2006), a reaction that can be monitored spectrophotometrically. Final conditions were 0.1M TEA, pH 7.9 containing 1.0 mM ATP, 33 mM $MgCl_2$, 20 mM glucose, and 0.75 mM NAD^+. Assays were performed in 96-well microtiter plate format in a GENios spectrophotometer (Tecan Group Ltd., Switzerland).

Tryptophan Quenching Assay of TbHK1

Glucose (20 mM), QCN (50 μM) and ATP (varying concentrations) were added individually and in combination to a solution (3 ml) of 0.1M TEA, pH 7.4. A scanning spectrofluorometer (QM-Y, Photon Technology International, Birmingham, NJ) was used to monitor emission from 300-550 nm after excitation of the lone Trp on TbHK1 (W177) at 280 nm. After acquiring background emission, TbHK1 (~1 μg) was added to the cuvette, mixed by inversion, and an emission scan performed. Using the Photon Technology International software, the area under the emission curves from 370-380 nm was integrated. Values were converted into the percent of Trp emission lost and plotted versus concentration of substrate/inhibitor using KaliedaGraph software version 4.03. Sigmoidal curves were fit to the plots and IC_{50} values were determined for the substrate/inhibitor by setting y equal to 50 and solving for x using the sigmoidal equation.

QCN Localization in T. brucei by Fluorescence Microscopy

PF parasites (29-13, a 427 strain) were grown in SDM-79 with the T7 RNA polymerase and the tetracycline repressor constructs maintained by the addition of 15 μg/ml G418 and 50 μg/ml hygromycin to the medium. BSF parasites (cell line 90-13, a 427 strain) were grown in HMI-9 supplemented with 10% fetal bovine serum and 10% Serum Plus (Sigma-Aldrich, St. Louis, MO) with the T7 RNA polymerase and the tetracycline repressor constructs maintained by the addition of of 1.5 μg/ml G418 and 5 μg/ml hygromycin to the medium.

For microscopic examination of QCN localization, *T. brucei* were grown to 1 x 10^7/ml (PF 29-13) or 1 x 10^6/ml (BSF 90-13), harvested (800 x g, 10 min), and washed twice in modified PBS (5 mM KCl, 8 mM NaCl, 1 mM $MgSO_4$, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 20 mM glucose). QCN (100 μM) was then added to cells in the modified PBS. After incubation (15 min, at growth conditions), cells were pelleted, washed twice, and applied to slides after the addition of VectaShield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). Images were captured by epifluorescence microscopy (Axiovert 200M, Carl Zeiss MicroImaging, Inc., Thornwood, NY).

For glycosome labeling, the aldolase peroxisomal targeting sequence (PTS2) (Blattner et al., 1995) was introduced into a red fluorescent protein (mCherry) modified pXS (Marchetti et al., 2000) expression vector to yield an N-terminal fusion with the mCherry. Briefly, FPTS2 (5'AGCTTATGAGTAAGCGTGTGGAGGTGCTTCT TACACAGCTTG 3') and RPTS2 (5'CTAGCAAGCTGTGTAAGAAGCACCTCCAC ACGCTTACT CATA 3') were annealed and the resulting product cloned into pXS. PF parasites were then transiently transfected with 10μg of the pXSAldoPTSmCherry construct and cultured 24 hr prior to examination. Live cells were visualized after resuspension in mounting medium (with DAPI) diluted 1:1 in PBS.

For RNAi studies, PF parasites were transfected and stable transformants selected as described (Wang et al., 2000). TbHK1 was targeted specifically using an RNAi construct that targeted the unique 3'UTR of the transcript. Briefly, RNAi of TbHK1 was achieved using pZJM harboring a 341 bp fragment previously identified as a 3'

untranslated region sequence (Morris et al., 2002). Parasite growth was monitored on a Becton-Dickinson FACScan flow cytometer.

For studies exploring the impact of over-expression of TbHK1 in PF cells, parental cells (PF 29-13) were transformed with linearized pLew111(2T7)GFPb (Motyka et al., 2006) harboring the TbHK1 gene in the multicloning site. This vector fuses the green fluorescent protein (GFP) to the carboxyl termini of expressed proteins. After selection for stable transformants, TbHK1 expression was induced by addition of tetracycline (1 μg/ml) to the media.

RESULTS

Glycolysis is essential to the parasitic protozoan *T. brucei*, suggesting that inhibitors of enzymes in the pathway may be suitable targets for therapeutic development. TbHK1, which mediates the first step in this metabolic pathway, is an enzyme that has previously demonstrated to be essential for BSF parasites (Chambers et al. 2008a). As part of the validation of a high throughput screening campaign, we completed a pilot screen of a library of 1280 pharmaceutically active compounds (LOPAC, Sigma) (Sharlow et al, 2009.). This screen yielded 12 primary hits including myricetin (IC_{50} of 48.9 ± 0.7 μM), a bioflavonoid that shares structural similarity with a known anti-trypanosomal compound, QCN (Fig. 4.1A) (Mamani-Matsuda et al., 2004). Further, the observation that QCN inhibited mammalian HKs (Graziani, 1977) suggested that the trypanosome TbHK1 could be a target of QCN. These observations led us to

further explore QCN as an inhibitor of TbHK1 while considering the possible connection between TbHK1 inhibition and the reported anti-parasitic activity of QCN.

QCN inhibits TbHK1 through mixed inhibition with ATP

Incubation of recombinant TbHK1 with QCN followed by a coupled enzyme assay for HK activity revealed that the compound inhibited the enzyme (IC_{50} = 4.1 ± 0.8 μM) (Fig. 4.1B). Inhibition was not as a result of dissociation of TbHK1 oligomers (a previously characterized mechanism for regulation of activity (Chambers et al., 2008)), as QCN did not cause dissociation of TbHK hexamers.

Many different kinases are inhibited by QCN, indicating that the molecule interacts with a structural feature common to all of the proteins, with the ATP binding site being a likely candidate binding site (Matter et al., 1992; Srivastava, 1985; Granot, 2002). Analysis of the nature of TbHK1 inhibition revealed that QCN was a mixed inhibitor with respect to ATP with a K_i value of 2.9 ± 0.9 μM (Fig. 4.1C).

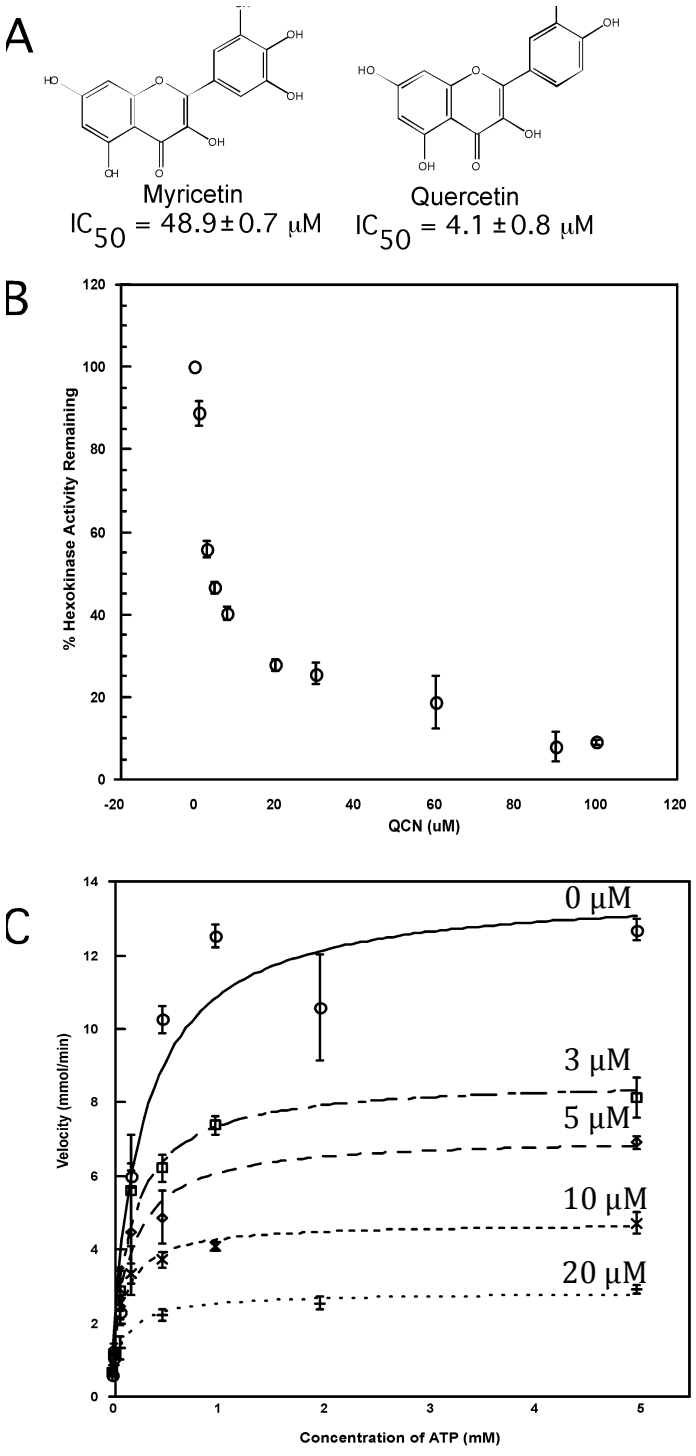


Figure 4.1. QCN is a potent inhibitor of TbHK1.

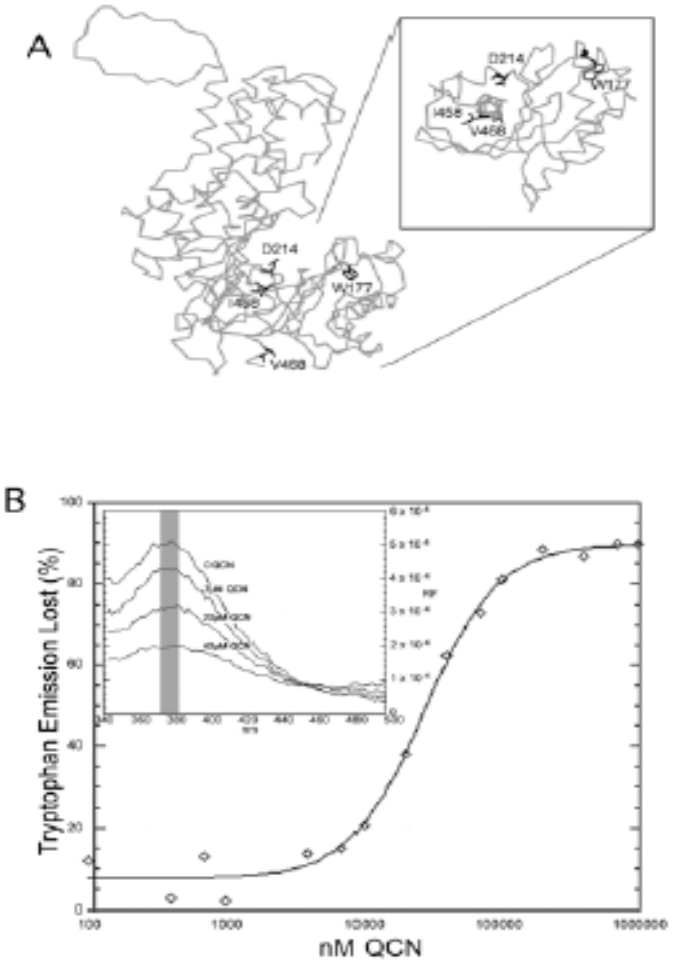
(A) Structures of myricetin and QCN. IC_{50}s for rTbHK1 are reported below the structure. (B) QCN inhibits rTbHK1. Increasing amounts (0-100 μM) of QCN were incubated with rTbHK1 (160 ng/assay) for 10 min at RT and HK assays were performed as described in the Materials and Methods. Please note that due to the complex nature of the mode of inhibition, IC_{50} values were estimated assuming single site binding. (C) QCN is a mixed inhibitor of TbHK1 with respect to ATP. Michaelis-Menten plots of inhibition with different QCN concentrations in assays containing varied ATP amounts.

To determine if endogenous HK activity from parasite lysates was also sensitive to QCN inhibition, cell lysates from BSF or PF parasites were incubated with QCN and then assayed for HK activity. QCN inhibited both BSF and PF HK activity similarly, with IC_{50}s = 24 μM and 30 μM, respectively.

TbHK1 inhibition coincides with changes in Trp177 fluorescence

TbHK1 harbors a single Trp (residue 177) that is modeled to lie on the face of the enzyme near the hinge region and catalytic base (Asp214) (Fig. 4.2A) (Morris et al., 2006). Excitation of the single Trp177 in TbHK1 at 280 nm yielded a characteristic Trp emission band at ~370 nm (Fig. 4.2B, inset). While QCN is intrinsically fluorescent with a maximum emission wavelength of 550 nm when excited at 280 nm, it alone yielded little fluorescence at 370 nm. Addition of increasing amounts of QCN to TbHK1, however, quenched the Tryp177 emission, yielding an IC_{50} for quenching of ~ 35 μM (Fig. 4.2B).

To further resolve the impact QCN was having on Trp177 emission, substrates of TbHK1 were included to determine their impact on fluorescence. Addition of glucose (20 mM) alone did not alter Trp177 emission at 370 nm (not shown). However, addition of ATP (0.05 mM) quenched the Trp177 emission ~20%, while additional ATP (to 5 mM) nearly eliminated emission (Fig. 4.2C). Addition of glucose with the ATP did not alter quenching, yielding emission loss similar to ATP alone.



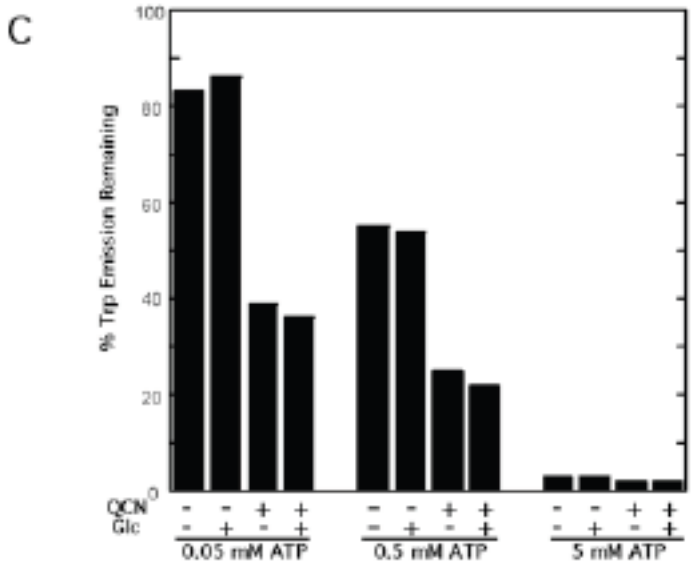


Fig. 4.2. QCN bound to rTbHK1 alters Trp177 fluorescence.

(A) TbHK1 was modeled to the crystal structure of *S. cerevisiae* hexokinase PII (Kuser et al., 2000). The active site, Asp214, and Trp177 are indicated, along with residues that are involved in ATP binding in the C-terminal tail. For clarity, the lower lobe of the protein has been isolated and rotated to position the C-terminal tail perpendicular to the page. (B) Trp177 emission is inhibited by QCN. TbHK1 (1 µg) was incubated with increasing concentrations of QCN in a 100 mM TEA (pH 7.4) solution and, following excitation at 280 nm, emission monitored from 340-500 nm. To assess the percentage of Trp emission lost, the region between 370-380 nm (inset) was calculated and the % reduction in response to QCN determined. (C) ATP and QCN quench Trp177. Spectra were acquired from samples containing different amounts of ATP as described in 2B, and percentage of Trp emission lost calculated. Glucose (20 mM) was added as indicated.

QCN localizes in part to glycosomes in live trypanosomes

Little is known about the mechanisms of action of QCN in the African trypanosome but the observation that the compound inhibits the essential TbHK1 led us to consider if the two shared subcellular localization. To explore this, live PF parasites expressing glycosomally targeted mCherry were incubated with QCN and the fluorescence both scored by microscopy (Fig. 4.3A). QCN accumulated in distinct foci, yielding punctate fluorescence that co-localized with the mCherry-bearing glycosomes. Additional QCN fluorescence was observed as a light haze throughout the cell, suggesting that localization was not limited to glycosomes. (Please note, parasite autofluorescence was not observed when similar exposure times were used on cells not incubated with QCN, suggesting the diffuse signal is due to the compound.) Similarly, live BSF parasites yielded QCN fluorescence in punctate foci, a distribution suggesting subcellular localization, potentially glycosomal in nature (Fig4. 3A). Additional staining was observed associated with the flagellum.

The glycosomes, a peroxisome-like organelle, compartmentalizes the majority of the glycolytic enzymes in the trypanosome, many of which have been demonstrated genetically to be essential, including TbHK1 (Chambers et al., 2008). The localization of a compound that inhibits TbHK1 *in vitro* to the compartment that houses the essential enzyme perhaps explains the cytotoxicity of the compound. Previous research demonstrated that QCN is toxic to *T. b. gambiense* (LD_{50} = 10 μM) (Mamani-Matsuda et al., 2004). To confirm that our 427 *T. b. brucei* lab strain was also susceptible to QCN, parasites were incubated for 24 hr in the presence of compound and growth monitored by

cell counting. QCN was found to be toxic to both BSF 90-13 and PF 29-13 trypanosomes (LD_{50} = 7.5 μM and 35 μM, respectively).

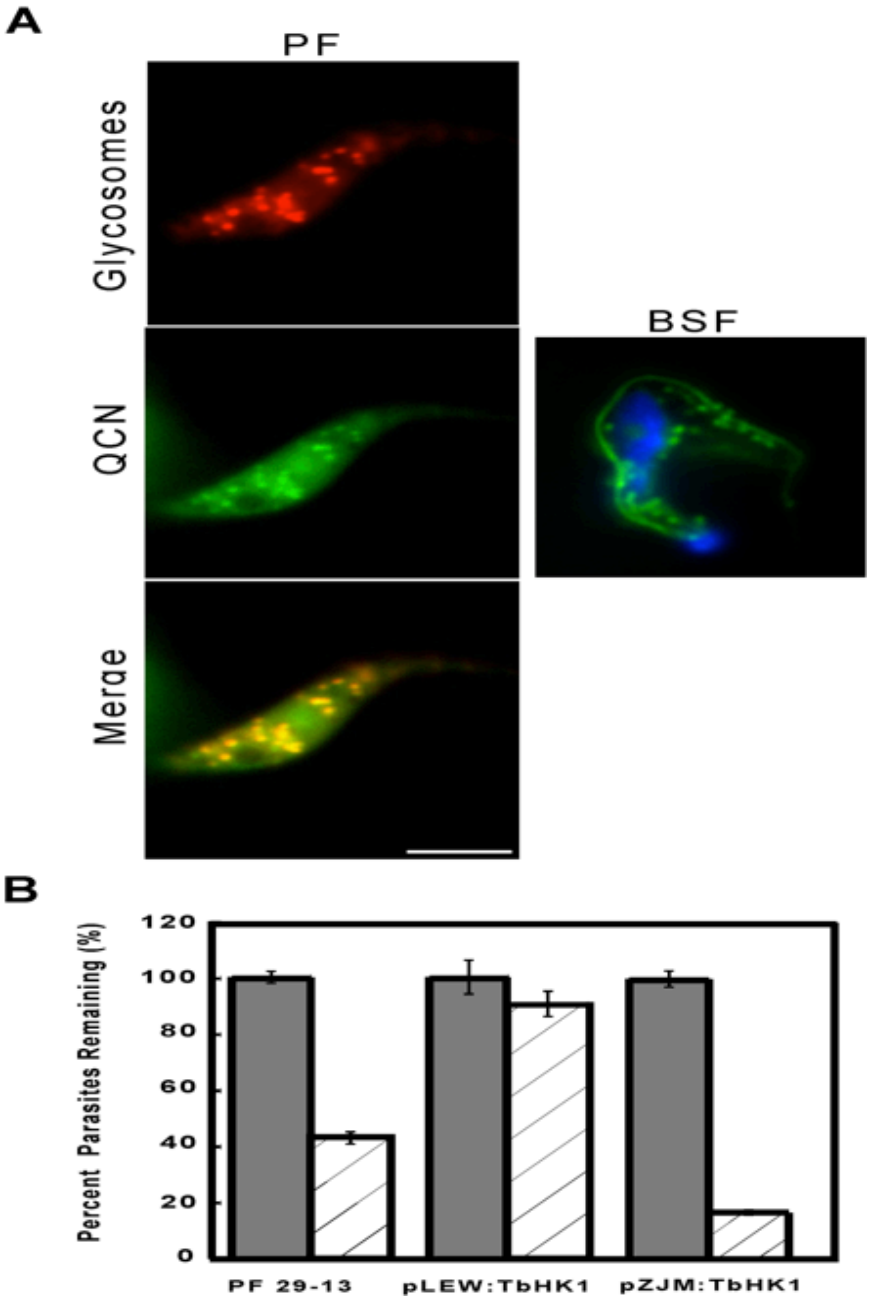


Figure 4.3. Localization and genetic manipulation studies to explore the biological consequences of QCN on *T. brucei*.

BSF and PF parasites incubated with QCN (100 μM, 15 and 10 min for PF and BSF, respectively) were stained with DAPI and imaged. Glycosome localization in live PF cells was visualized by mCherry emission. Fluorescence of the QCN does not bleed into the mCherry spectra and was captured using a 488 nm filter set. Scale bar = 10 μm. (B) RNAi of TbHK1 enhances QCN toxicity, while over-expression of TbHK1 tempers it. Parental PF trypanosomes (29-13) or parasites transformed with pLEW111(2T7):TbHK1 or pZJM:TbHK1 were induced with tetracycline (1 μg/ml) for 24 hours to either express or silence TbHK1, QCN (50 μM, dashed columns) added, and cell viability scored after an additional 24 hour incubation. All assays were performed in triplicate, with cell numbers normalized to untreated controls. The p-values for untreated and treated parental 29-13 and pZJM:TbHK1 bearing cells were statistically significant ($p < 0.05$ in both cases), indicating that the differences between untreated and treated cells were significant, while the value for pLEW111(2T7):TbHK1 harboring cells incubated with or without QCN was not ($p > 0.05$)

Exploring potential in vivo *targets of QCN*

Unlike BSF parasites (which rely exclusively on glycolysis for ATP production), genetic manipulation of glycolytic enzymes, including over-expression and RNAi-based silencing, can be tolerated in PF parasites if the trypanosomes are first provided an opportunity to down-regulate hexose metabolism (Morris et al., 2002; Morris et al., 2006). If QCN is toxic as a result of its inhibition of glycolysis, increased cellular TbHK1 polypeptide could temper the toxicity of the compound. To explore this, PF cells over-expressing TbHK1 from the inducible ectopic expression vector pLew111(2T7) were incubated with 50 μM QCN and cell growth compared to parental cell lines grown

under similar conditions (Fig. 4.3B). After 24 hrs, cells over-expressing TbHK1 displayed cellular HK activity 2.1 greater than control cells. Additionally, these cells were more resistant to QCN with growth reduced only 10.1 ± 0.5% while growth of QCN-treated control parasites was repressed 55.5± 0.5%.

The incomplete penetrance of RNAi (and the slowly developing phenotypes associated with the silencing) allows manipulation of TbHK1 abundance without detectable cell toxicity in PF parasites (Morris et al., 2002). Therefore, we have explored the consequence of silencing TbHK1 on QCN sensitivity, with the prediction being that these cells may be more sensitive to the compound (that is, the remaining TbHK1 that results from incomplete penetrance will be inhibited more readily by QCN). RNAi of TbHK1 using pZJM (TbHK1), which targets the distinct 3'UTR of *TbHK1* (Morris et al., 2002), was induced for 24 hours before cells were passed into medium containing QCN and cell growth compared to parental PF 29-13 parasites after an additional 24 hours. Silencing TbHK1 led to enhanced sensitivity to QCN, with cells induced to silence TbHK1 reduced in number to 16.4 ± 0.6% of the corresponding untreated cell lines (Fig. 4.3B).

DISCUSSION

BSF *T. brucei* generate ATP exclusively by glycolysis, indicating that inhibitors of enzymes in the pathway may be therapeutic lead compounds. The trypanosome glycolytic enzymes, TbHK1 and TbHK2, are potential drug targets. These proteins, which are 30-33% identical to those from yeast, plants, and mammals, have a number of

unique biochemical features (in addition to amino acid composition) that suggest that identification of trypanosome-specific HK inhibitors may be possible. These differences include mulitmerization of the enzyme into hexamers, localization of the protein to the glycosome, and sensitivity of the TbHKs to fatty acids (Chambers et al., 2008; Misset and Opperdoes, 1984; Morris et al., 2006).

Genetic studies using RNAi have shown that both TbHKs are essential to the parasite (Albert et al., 2005; Chambers et al., 2008), and chemical inhibitors of the enzyme have demonstrated anti-parasitic activity *in vitro* (Chambers et al., 2008; Sharlow et al.; Willson et al., 2002). Here, we report that QCN, a previously recognized anti-trypanosomal flavanoid (Mamani-Matsuda et al., 2004), inhibits recombinant TbHK1. Amongst the mammalian enzymes that have been reported to be sensitive to QCN, including a tyrosine protein kinase, a phosphorylase kinase (Srivastava, 1985), a phosphatidyl 3-kinase (Matter et al., 1992), and a DNA topoisomerase (Boege et al., 1996), the IC_{50}s for QCN are similar to that of TbHK1, ranging from 3-300 μM. This suggests that there may be limited increase in therapeutic range for the compound. However, the observation that QCN is not toxic to mammals at trypanocidal concentrations (Hu et al., 2001) supports the possibility that the partial localization of QCN to glycosomes (and the potential for concentration of the compound therein) may increase the therapeutic ratio favorably.

Many of the other enzymes that have been reported to be sensitive to QCN share the feature of binding nucleotides or nucleotide triphosphates. It has been suggested that QCN inhibits Tyr kinases (like $pp60^{v\text{-}src}$) by forming a hydrogen-bonded complex with

ATP, which would mimic the transition state of ATP and the tyrosyl residue of the enzyme (Granot, 2002). A similar mechanism is unlikely in TbHK1, as QCN alone can quench Trp177 fluorescence.

QCN has proven to be a potent anti-kinetoplastid agent, with toxicity toward *Leishmania donavani* (Mittra et al., 2000) and *T. brucei gambiense* (Mamani-Matsuda et al., 2004). In the former case, QCN inhibits DNA synthesis, leading to cell cycle arrest that triggers apoptosis. Similarly, QCN causes *T. brucei* death through apoptosis (Mamani-Matsuda et al., 2004), though we propose the mechanism may be distinct. In mammalian cells, mitochondrial associated HK activity is required to prevent apoptosis (Gottlob et al., 2001) and increased HK activity prevents oxidant-induced apoptosis (Bryson et al., 2002). Our observation that QCN inhibits TbHK1 suggests that the apoptosis may be the result of a mechanism that measures HK activity, similar to that found in mammalian cells. The TbHKs are unusual in a number of ways, including the finding that they oligomerize into hexamers (Chambers et al., 2008) and localize to the peroxisome-like glycosome (Misset and Opperdoes, 1984). These characters suggest novel mechanisms connecting TbHK to cell signaling, which remain to be resolved.

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CHAPTER 5

EXPRESSION OF *TRYPANOSOMA BRUCEI* HEXOKINASE 1 IS REGULATED BY A METAZOAN-LIKE MICRORNA-BINDING ELEMENT

Heidi C. Dodson, Andrew C. Sayce, Meredith T. Morris, and James C. Morris*

Department of Genetics and Biochemistry

Clemson University

Clemson, SC 29634

ABSTRACT:

Hexokinase activity is essential in *Trypanosoma brucei* and tightly regulated as small changes in expression level have dramatic consequences to cells. *T. brucei* expresses two nearly identical hexokinases (*TbHK1* and *TbHK2*) in both procyclic (PF) and bloodstream (BSF) form parasites. While the TbHKs share 98.5% identity at the nucleotide level from the 5'UTR through the ORF, the 3'UTRs are distinct, suggesting regulation of expression may be controlled by 3'UTR elements. Using a deletion strategy to remove portions of the TbHK1 and TbHK2 3'UTRs, we have identified regions in the 3'UTRs that are responsible for conferring regulation of expression. Of note, the 3'UTRs of both genes contain putative K-box and CAAC elements. In *Drosophila*, these elements bind microRNAs and function as negative expression regulators. To determine if the putative K-box/CAAC elements are involved in differential regulation of expression of TbHKs, we assessed the impact of point mutations in the K-box of both the

TbHK1 and TbHK2 3'UTRs on reporter expression. While a T to G point mutation in the TbHK1 K-box led to a 6-fold increase in expression, a similar change in the TbHK2 3'UTR K-box had no effect. Additionally, co-transfection of a synthetic anti-sense RNA, asRNA, (to mimic the possible K-box binding partner) restored TbHK1 expression levels suggesting that the K-box/CAAC motif may be an important element in regulating expression of TbHK1.

INTRODUCTION

T. brucei, the causative agent of African sleeping sickness, does not execute conventional eukaryotic mechanisms of transcription. The protein coding regions of trypanosomatid genomes are organized into polycistrons containing up to several hundred unrelated genes (Haile and Papadopoulou, 2007). Transcription is accomplished in a polycistronic fashion with every gene located on the polycistron being transcribed at the same time. The long precursor RNAs are then processed into mature mRNAs by a coupled *trans*-splicing and polyadenylation reaction (Sutton and Boothroyd, 1986; LeBowitz et al., 1993). The paucity of transcription factors and RNA polymerase II promoters in the genome and small percentage of differentially regulated genes noted in global gene expression profiling during development suggest that gene regulation is accomplished primarily post-transcriptionally (Clayton, 2002).

Post-transcriptional mechanisms of gene expression regulation in *T. brucei* include a number of *cis*-acting elements found in the 3'UTRs of the regulable mRNA (Clayton, 2002; Haile and Papadopoulou, 2007). These elements generally influence the mRNA stability or translational efficiency of the transcript (Ouellette and Papadopoulou, 2009). Two of the most well characterized regulatory elements are found in the 3'UTR of procyclins, a U-rich 26-mer that confers mRNA stability and a16-mer that enhances translation in procyclic form parasites (Hehl et al., 1994).

Regulation via 3'UTR elements likely requires interaction with *trans* regulatory molecules that influence the stability or translation of the transcript. For example, the *Trypanosoma cruzi* U-rich binding proteins bind to mucin mRNAs and regulate their

stage specific expression (D'Orso and Frasch, 2002). In most other eukaryotes, small RNAs also serve as *trans*-regulators of gene expression. These small RNAs include microRNAs (miRNA) and antisense RNAs (asRNA). While computational efforts suggest that *T. brucei* may harbor a number of miRNAs, these have yet to be isolated (Mallick et al., 2008). Similarly, asRNA has not been identified in *T. brucei*, though *Leishmania* expresses sense and anti-sense forms of non-coding RNAs (Dumas et al., 2006). The parasite does harbor the machinery to produce small RNAs, as demonstrated by a robust RNA interference response. The role of this system, however, may be limited to one of genome protection from retrotransposable elements (Ullu et al., 2004).

T. brucei express two hexokinases, TbHK1 and TbHK2, in both the insect form and bloodstream form of the parasite (Colasante et al., 2006). *TbHK1* and *TbHK2* are located in tandem on chromosome 10 and encode proteins that are essential to the parasite (Morris et al., 2002; Morris et al., 2006; Berriman et al., 2005). Little is known about the regulation of expression of these genes. While the putative 5'UTRs and ORFs are 98.5% identical at the nucleotide level, the 3'UTRs are distinct, suggesting that regulatory information could be housed in the 3'UTRs. Here we demonstrate the role of a metazoan-like microRNA-binding element in the regulation of *TbHK1* expression, and present evidence that a novel asRNA is involved in the process.

MATERIALS AND METHODS

Cell Culture and Transfections - PF parasites were grown in SDM-79 (Brun et al., 1979) and quantified prior to transfection. A total of ~ 1.0 x 10^8 cells were transfected via electroporation (ECM 830 Electroporator, BTX, San Diego, CA) with 10 µg of plasmid DNA. After electroporation, cells were cultured in SDM-79 (28°C, 5% CO_2) and CAT assays were conducted after a 24 hour recovery period. Bloodstream form (BSF) parasites were grown in HMI-9 media (37°C with 5% CO_2) and transfected. After electroporation, BSF parasites were allowed to recover for 24 hours (37°C, 5% CO_2) prior to lysing for assessment of CAT activity.

Cloning of constructs for CAT Assays – Presumptive 3'UTRs were amplified by PCR from *T. b. brucei* genomic DNA and products cloned into pGEM (Promega, Madison, WI) and sequenced. The *TbHK1* 3'UTR was amplified using F.TbHK1 3'UTR.BamHI (5' GATCGGATCCGTGAGAAAACCATGCTTTTC 3') and reverse primer R.TbHK1 3'UTR.XbaI (5' GATCTCTAGATATATATACCCTTTATTTGC 3'). TbHK2 3'UTR was amplified with F.TbHK2 3'UTR.BamHI (5' GATCGGATCCAGGTGA GTGTCCGCGAACG 3') and R.TbHK2 3'UTR.XbaI (5' GATCTCTAGATAACCG CTTGGATTCCTATTTTTC 3'). The pGEM inserts were subsequently used to replace the EP1 procyclin 3'UTR in pGAPRONE (Furger et al., 1997). This construct drives expression of the chloramphenicol acetyl transferase (CAT) gene from a procyclin promoter and was the generous gift of Drs. Erik Vassella and Isabel Roditi (University of Bern). Deletion constructs were generated using a Phusion Site-Directed Mutagenesis

Kit (Finnzymes, Woburn, MA). The K-box point mutants were created by site directed mutagenesis (QuikChange II Site Directed Mutagenesis kit, Stratagene, La Jolla, CA).

CAT Assays - CAT activity was determined using a colorimetric assay with 5'-dithiobis-(2 nitrobenzoic acid) (DTNB) as indicator. In short, 24 hr after transient transfection, cell amounts were quantified, collected by centrifugation (3,000 rpm, 10', RT) and lysed with water. Acetyl-CoA (100µM final concentration/reaction) was added to an optically clear 96 well plate with cell lysate followed by sequential addition of chloramphenicol (100µM final concentration) and DTNB (0.5 µM final). In brief, CAT activity is assessed using a colorimetric assay. The assay measures CAT activity via the transfer of the acetyl group from acetyl-CoA to chloramphenicol. This causes the which release of a thiol group t that then reacts with DTNB to release 2-nitro-5-mercaptobenzoic acid (TNB), which can be monitored spectrophotometrically at 405nm.

mfold – mfold analyses were conducted using the mfold web server (version 3.2) in batch preparation (Matthews et al., 1999). Conditions were set to default (linear RNA, 37°C, 1M NaCl, no divalent ions, 5% suboptimality, maximum interior bulge/loop size and asymmetry 30 nt, no limit on maximum distance between paired bases). The structure presenting the lowest initial dG was used for all subsequent analysis.

RNA cloning and* in *vitro transcription - A synthetic anti-sense RNA (asRNA) was generated using primers F.TbHK1 3'UTR 1077.920RC.XhoI (5' CACCTCGAGT CATTCATCTCCATTC 3') and R.TbHK1 3'UTR 1077.920RC.HindIII (5' GTGAAGC

TTGAGGAGAGGAATCATA 3')to amplify the reverse strand of the *TbHK1* 3'UTR from previously synthesized cDNA. This product was subcloned into the pZJMα vector containing dual T7 promoters (Wang et al., 2000). Following *Hind*III linearization of the vector, asRNA *in vitro* production was driven from the T7 promoters using an AmpliScribe T7 High Yield Transcription Kit (Epicentre Biotechnologies, Madison, WI).

RESULTS

Assessment of Basal Levels of Expression of TbHK1/2

To assess differential expression of TbHK1 and TbHK2 in BSF and PF parasites, a reporter gene system was employed to evaluate ratios of basal expression levels of these two almost identical proteins. Putative sequences for both the TbHK1 3'UTR and TbHK2 3'UTR, 1297nt and 500nt, respectively, were cloned downstream of a CAT reporter gene. Expression levels were quantified relative to TbHK1 3'UTR.

Previously, analysis of steady-state mRNA levels suggested that TbHK1 transcript was ~2-fold higher than TbHK2 (Morris et al., 2006). In good agreement with this observation, the expression from the CAT/TbHK1 3'UTR construct was about twice that from the CAT/TbHK2 3'UTR construct in procyclic form parasites (Fig. 5.1). It should be noted, however, that expression levels do not necessarily directly corelate with transcript levels, especially in an organism that regulates all gene expression post transcriptionally. Differential expression was less robust in BSF parasites, with TbHK1 expressed ~20% higher than TbHK2 (Fig. 5.1).

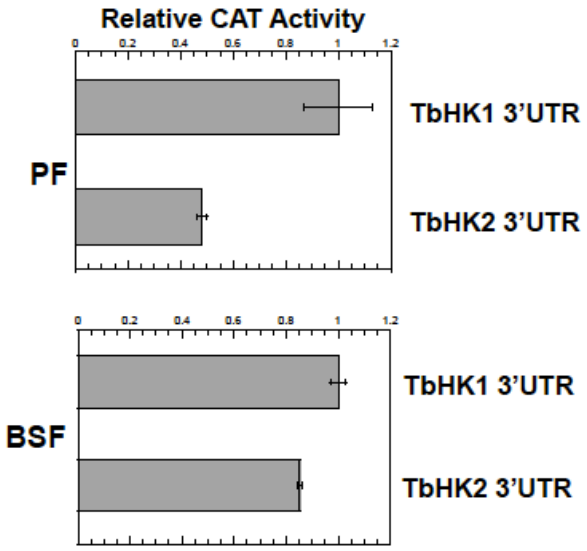


Figure 5.1. Differential expression of TbHK1 and TbHK2 in PF and BSF. CAT activity was assessed using putative full length CAT reporter constructs-TbHK1 3'UTR (1077 nt) and TbHK2 3'UTR (500 nt) in PF (top) and BSF (bottom) parasites.

Deletion Mapping of 3'UTR Regulatory Elements

Further, to identify putative regulatory elements involved in the expression of *TbHK1* and *TbHK2*, deletion constructs were generated by removing portions from the proximal end or internal portion of each 3'UTR (Fig. 5.2). Deletion of the proximal 300 nt of the TbHK1 3'UTR ablated expression, suggesting that this region of the transcript harbors a positive regulatory region (Fig. 5.2A). Additionally, removal of the proximal 400 nt yielded expression levels less than full length, exposing a positive regulatory element located between 300-400 nt. Removal of 600 nt from the UTR yielded ~1.5-fold higher expression. These data together suggest a negative regulatory element located

between 300-600 nt of the UTR. Overall, the deletions revealed that the TbHK1 3'UTR harbors several regulatory sequences to adequately regulate expression (Fig. 5.2A).

Deletion analysis of the TbHK2 3'UTR suggested the presence of a negative regulatory element, though the mechanism is probably distinct from that of TbHK1 (Fig. 5.2B). Deletion of the first 200, 300, or 400 nt only led to modestly increased expression.

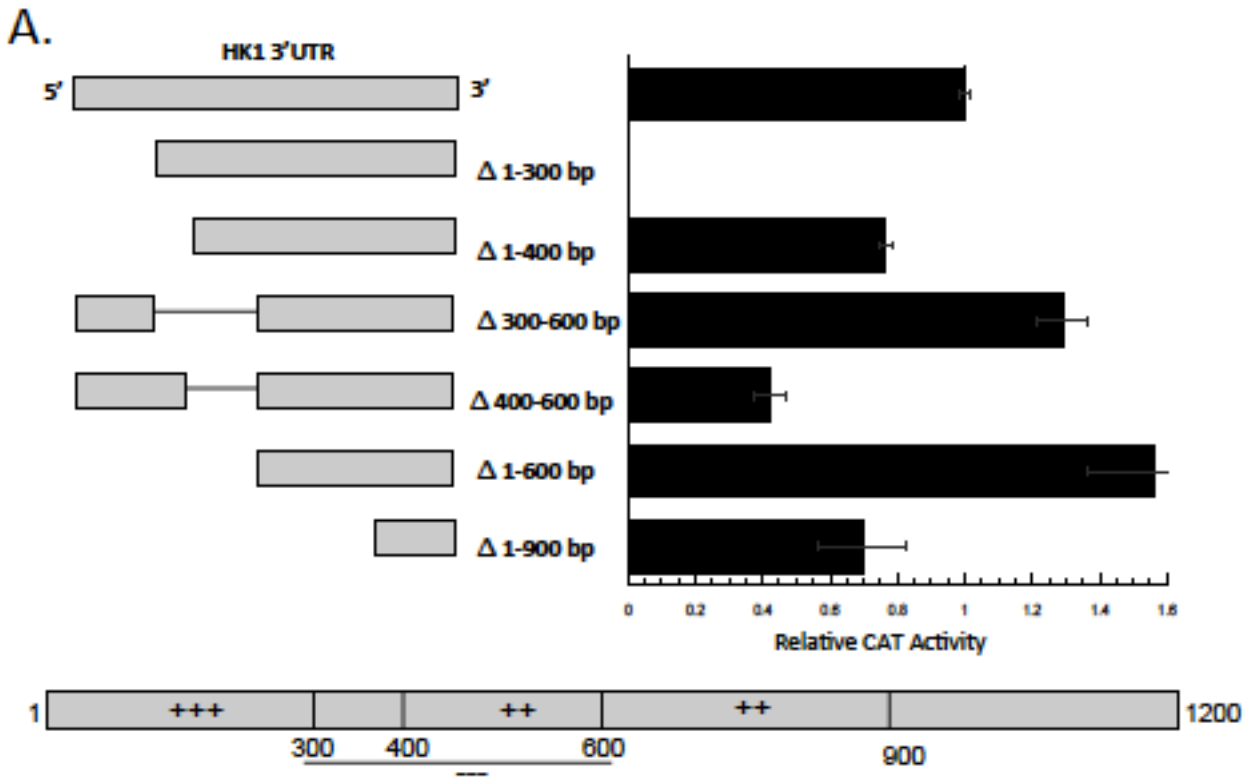


Figure 5.2. Mapping regulatory elements using truncation of the TbHK 3'UTRs fused to a CAT reporter.

CAT reporter gene activity was assessed in constructs bearing deletions of either the TbHK1 3'UTR (A) or TbHK2 3'UTR (B-see next page). A schematic representing regulatory elements identified from these studies is represented below each activity graph.

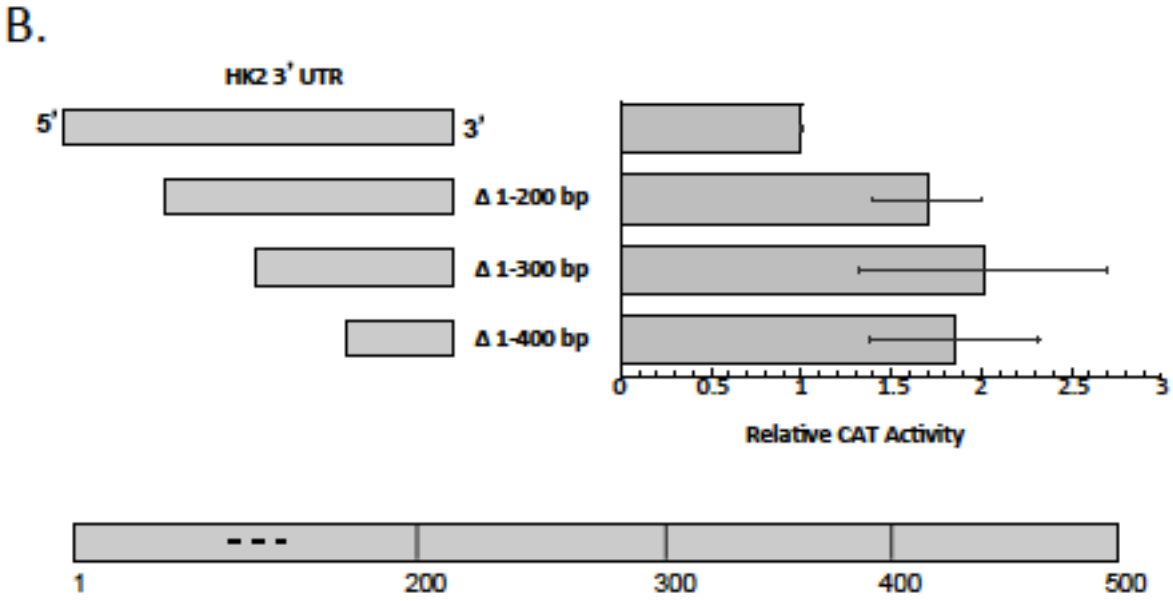


Figure 5.2. (cont.) Mapping regulatory elements using truncation of the TbHK 3'UTRs fused to a CAT reporter.

The 3'UTRs of TbHK1 and 2 harbor potential regulatory elements

Deep sequencing of PF trypanosomes has revealed that the 3'UTR of *TbHK1* contains seven polyadenylation sites following the stop codon, while the 3'UTR of *TbHK2* contains only one (Siegel et al., 2009). The seven lengths of the *TbHK1* UTRs range from 1026 nt to 1352 nt. The core region of the UTR (the first 1026 nts) harbor proximal (nt 430-444) and distal (nt 992-1005) K-box (cTGTGATa) and CAAC motifs (Fig. 5.3A) (Lai et al., 1998). These elements mediate negative post-transcriptional regulation in *Drosophila* (and likely other metazoa) (Lai et al., 1998), and serve as a likely mircoRNA binding site (Lai, 2002). In the *TbHK1* 3'UTR proximal K-box/CAAC motifs, the sequence and spacing between the K-box and CAAC elements is similar to that found in K-box/CAAC motifs from metazoans (Lai et al., 1998). The distal set of motifs, however, lacks conservation of the sequence and spacing, with the CAAC motif 5' to the K-box. RNA m-fold analysis (Matthews et al., 1999) of the *TbHK1* 3'UTR suggests that the K-box motifs are located on the same loop as their respective CAAC element (Fig. 5.3B).

The 353 nt of the *TbHK2* 3'UTR contains a core K-box element (TGTGAT) at nts 150-155 with a potential CAAC motif located 40 nts downstream (nt 196-199) (Fig. 5.3B). The *TbHK2* 3'UTR lacks the distal K-box/CAAC motifs. RNA mfold analysis suggests that unlike the proximal K-box/CAAC motif in TbHK1, these sequences in the TbHK2 3'UTR reside on spatial separate loops of the mRNA (Fig.5.3B)

A.

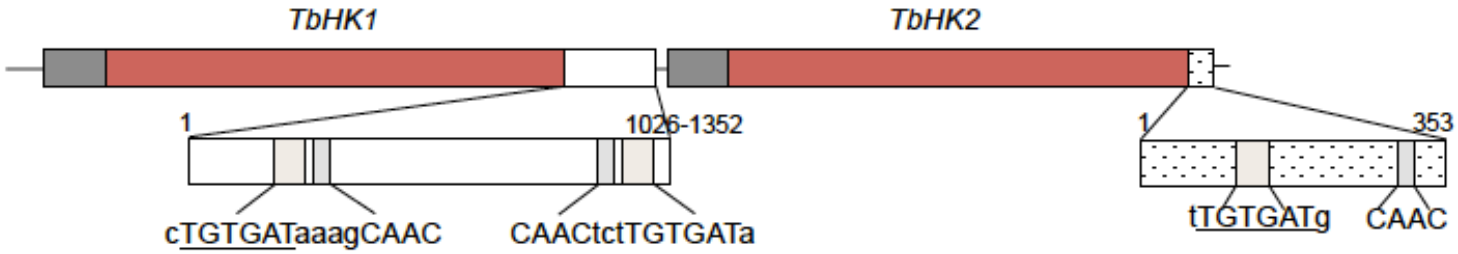
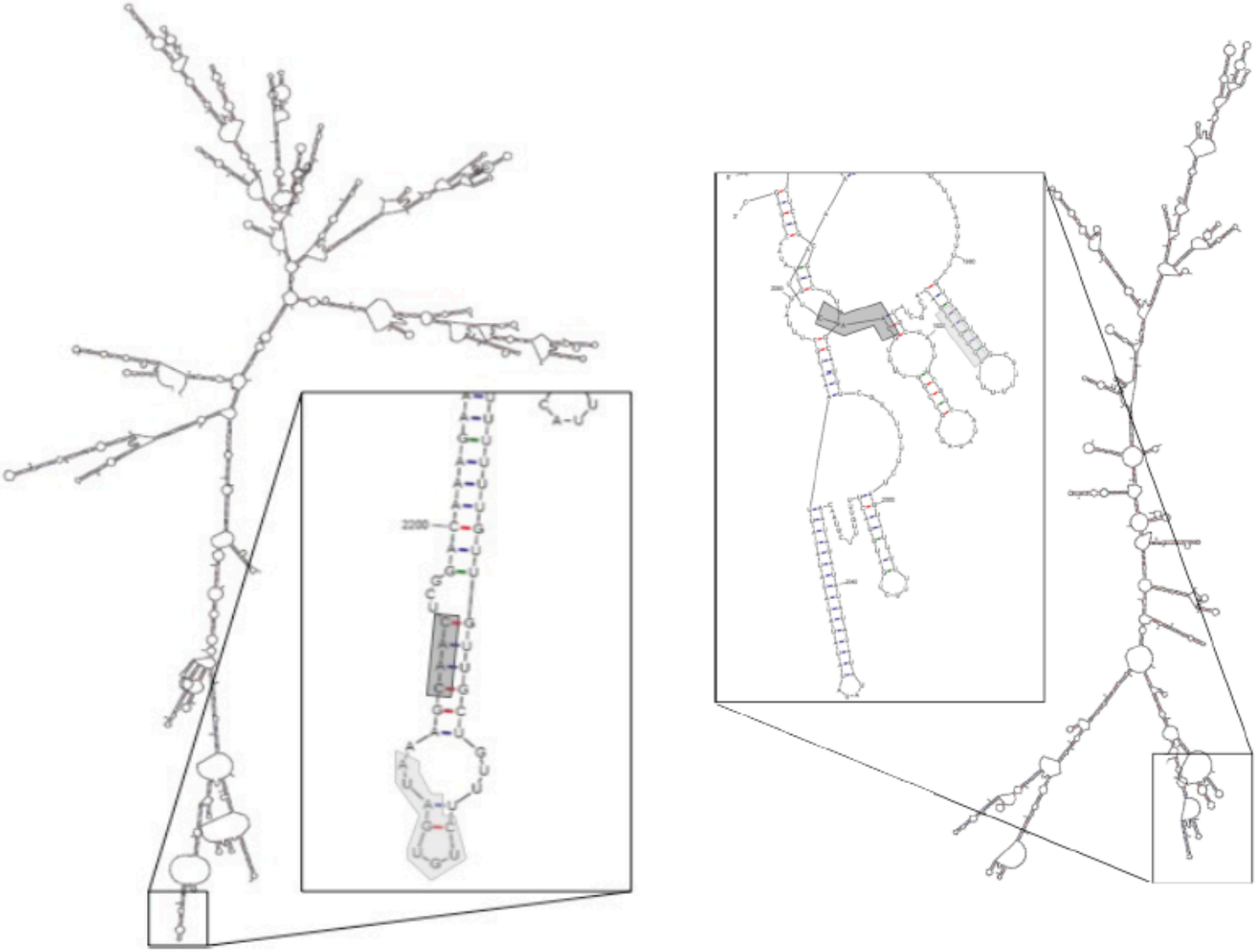


Figure 5.3. Tandem arrangement of TbHK genes along chromosome 10 highlighting UTR features.

(A) The 5'UTRs (black) are defined by a region of high (>98%) conservation preceding the open-reading frames. Hashed regions indicate ORFs with the highly variable 3'UTRs in white. Putative K-box motifs are shaded in dark gray with nearby CAAC motifs in light gray. Sequence data is given below each motif with conserved sequences underlined. (B) M-Fold analysis of the predicted structures of the 3'UTRs for TbHK1 (Left) and 2 (Right).

Figure 5.3B



Mutating the TbHK1 K-box alters expression

Deletion analysis suggested that the region of the TbHK1 3'UTR that harbors the K-box and CAAC motifs may play an important role in regulation of TbHK1 expression. To further assess this, a single nt change (T 431 to G) was made in the proximal K-box of the TbHK1 3'UTR. This change led to a ~6-fold increase in CAT expression (Fig. 5.4A). A similar change in the TbHK2 3'UTR K-box (T 150 to G) had no impact on TbHK2 expression (Fig. 5.4A).

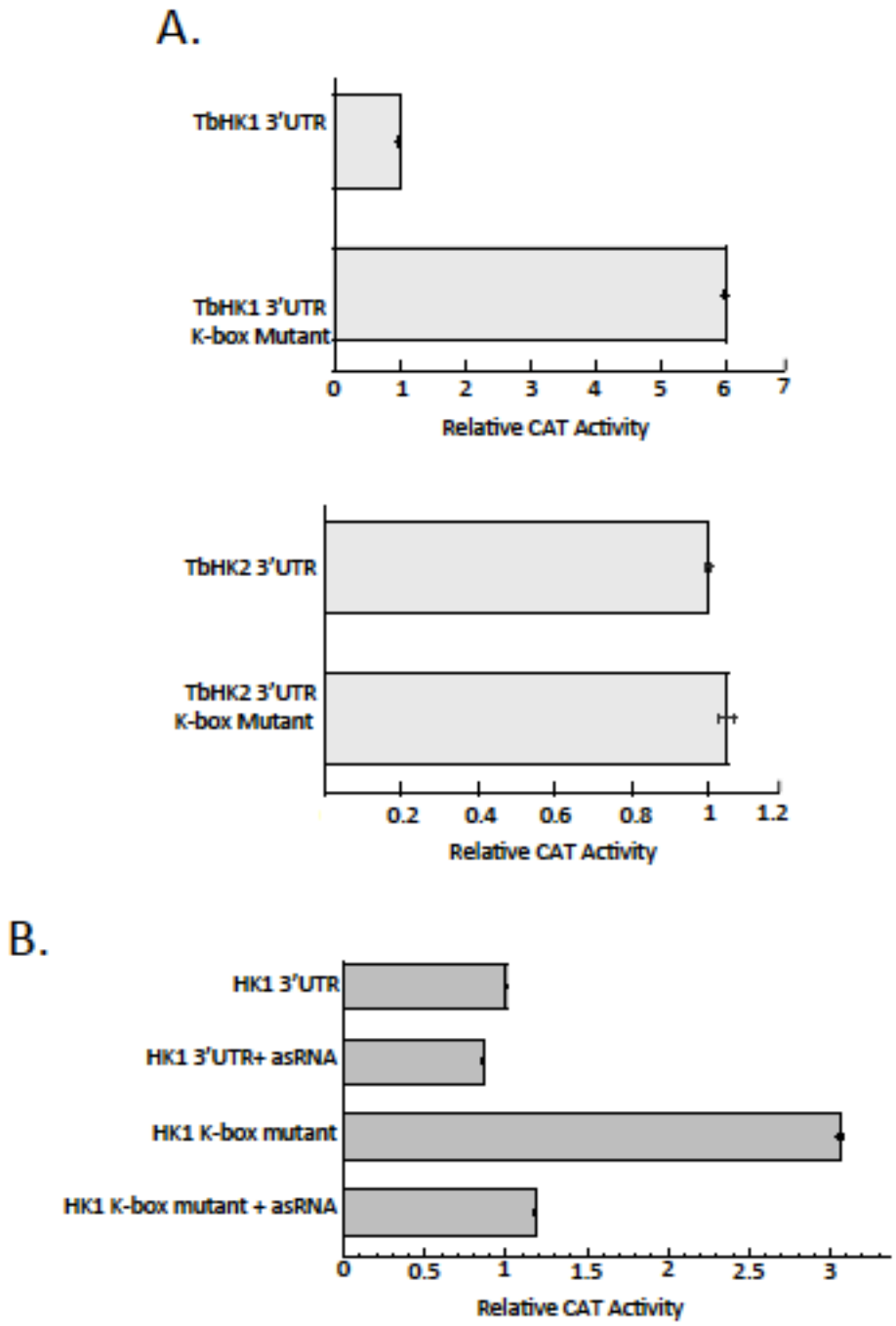


Figure 5.4. CAT enzyme reporter assays indicate that the proximal TbHK1 K-box is a negative post-transcriptional regulator.

(A) Mutating the proximal TbHK1 K-box (top) results in ~six fold increase in CAT reporter enzyme activity. Mutating the K-box of TbHK2 does not result in a change in CAT reporter activity (compared to native TbHK2 3' UTR sequence). (B) Co-transfection of TbHK1 3' UTR asRNA 920.1077 with CAT reporter constructs. TbHK1 3' UTR native and mutant K-box CAT reporter constructs were transfected or co-transfected with asRNA into PF parasites. Activity assays demonstrate minimal change for native TbHK1 3' UTR co-transfected with asRNA; however, the large increase in expression observed for mutant proximal K-box TbHK1 3' UTR CAT constructs is restored to approximately basal expression levels when asRNA is introduced.

In an effort to further understand the mechanisms involved in the K-Box motif-based regulation, we introduced the CAT/TbHK1 3'UTR constructs into an RNAi-deficient background using a *TbAgo*-deficient cell line (the kind gift of Dr. E. Ullu, Yale University). These results from these experiments were difficult to interpret, as regulation of all of the reporter constructs, including the EP13'UTR control construct, was unlike the parental control line PF29-13 (Fig. 5.5).

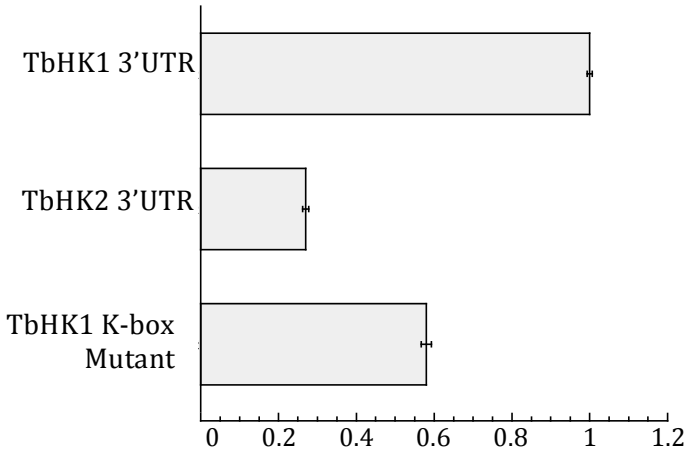


Figure 5.5 CAT Expression Gene Assays in TbAgo-deficient cell line.

TbHK1 3'UTR (1297 nt) and TbHK2 (500 nt) and TbHK1 3'UTR K-box Mutant constructs were transiently transfected into Ago-/- cells. CAT protein expression is reported relative to the TbHK1 3'UTR construct.

Co-transfection of a synthetic asRNA rescues TbHK1 expression

In *Drosophila,* dme-*mir2* and dme-*mir11* family miRNAs have been identified that bind to K-box/CAAC motifs (Lai et al., 2004; Lai et al., 2005). The curious presence of the distal CAAC/K-box, with the elements in a reversed orientation when compared to layout of the proximal elements, suggested that transcription of the distal element could yield RNA with partial complementary to the proximal K-box/CAAC motifs. To explore this, we first generated *in vitro* a 157 nt RNA (nt 920-1077, asRNA) using sequence flanking the distal CAAC/K-box as template. The asRNA was then co-transfected with CAT reporter constructs and the impact on expression assessed.

Co-transfection of the asRNA with the full length CAT/TbHK1 3'UTR had minimal impact on expression, indicating that the asRNA was not triggering an RNA interference based repression of expression (Fig.5.4B). However, co-transfection with the reporter construct harboring the single nt change in the K-box (which led to a 3-fold increase in expression level) yielded a return to full length expression level. The co-transfection of the asRNA did not trigger changes in expression of unrelated genes, as CAT expression from a construct harboring the EP1 3'UTR was not altered by the presence of the asRNA. Additionally, the asRNA had no impact on expression of the CAT/TbHK2 3'UTR construct.

A native anti-sense RNA (aRNA) corresponds to the distal K box of TbHK1

While investigating the possibility that the K boxes of *T. brucei* are regulated by the same mechanism as the K boxes of other species (namely a non-coding RNA such as a miRNA), we probed for small RNAs which could contain a seed sequence complimentary to the K box. In doing so, we produced cDNA for a 157 nt transcript corresponding to the anti-sense strand of the TbHK1 3' UTR at the 920th nt of the 3' UTR and extending to nt 1077 (Fig. 5.6). Interestingly, the distal TbHK1 K box and CAAC motif are contained within this region on the sense strand, thus when a portion of this strand is transcribed, a perfect reverse complement seed sequence for a K box target is produced.

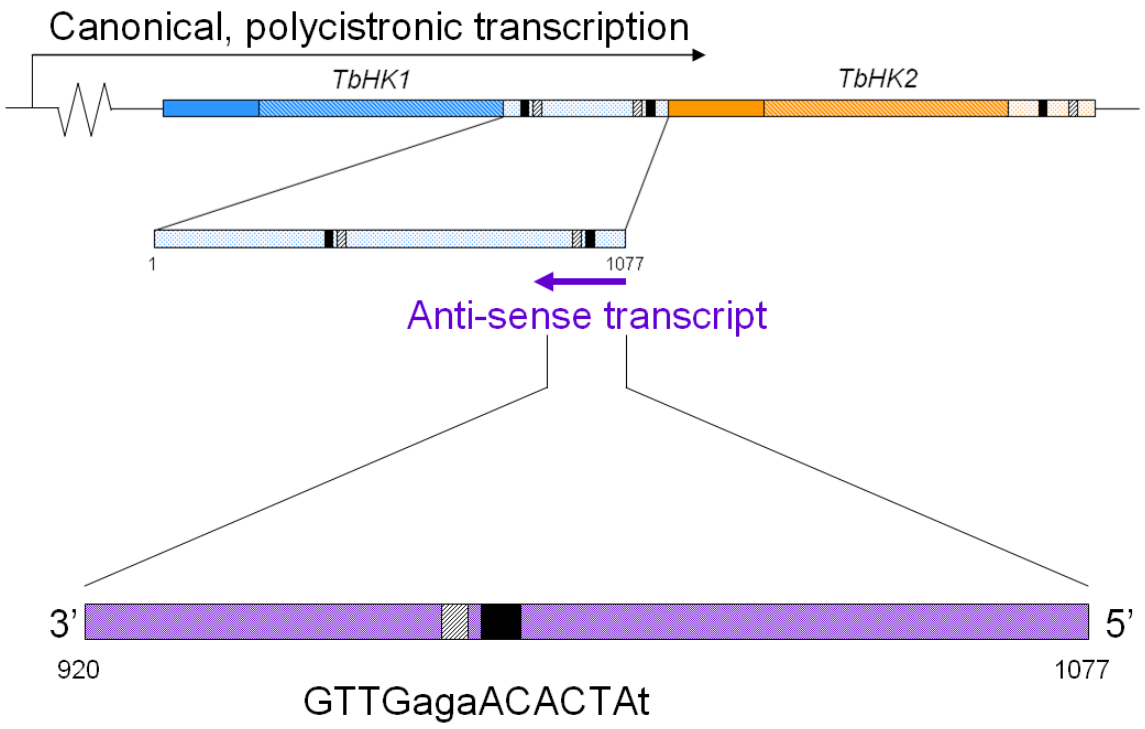


Figure 5.6. An anti-sense RNA (aRNA) is produced *in vivo* from the non-coding strand of the TbHK1 3' UTR.

The transcript detected by cDNA production includes at least the region from nt 920 to 1077 which harbors the distal K box and CAAC motifs. This transcript is produced from a DNA strand from which canonical polycistronic transcription in this 5' to 3' direction has not been identified in this region.

DISCUSSION

Transcription in *T. brucei* is accomplished in a polycistronic fashion with many unrelated genes being transcribed from the same polycistron (Haile and Papadopoulou, 2007). Therefore, most all gene regulation occurs post-transcriptionally in the parasite with regulatory sequences controlling mRNA stability and translation often located in 3'UTRs of genes. The parasite must be able to confer regulation in a rapid manner due to the extremely different environments encountered during its life cycle. One of the most striking differences observed when comparing life cycle stages of *T. brucei* are the metabolic pathways utilized for ATP production. This developmental difference involves the utilization of multiple metabolic pathways in procyclic form parasites to a sole dependence on glycolysis in bloodstream form parasites. Genes involved in these metabolic pathways, then, must be tightly regulated according to environment and subsequent nutrient availability. Here, we study mechanisms involved in regulating expression of the first enzyme in glycolysis, hexokinase.

T. brucei expresses two hexokinases in both PF and BSF, TbHK1 and TbHK2, that are 98.5 % identical at the nucleotide level. While the hexokinases exert little to no control over glycolytic flux (Albert et al., 2006), RNAi has demonstrated that both

TbHKs are essential (Morris et al., 2002; Albert et al., 2006) and transcript abundance of *TbHK2* demonstrates a marked increase in BSF parasites over PF parasites (Siegel et al., 2010) suggesting that life-cycle stage dependent expression of the hexokinases is regulated by the parasites. Further, our results indicate that study of TbHK post-transcriptional regulation may yield fundamental insights into trypanosomatid biology. The different roles of the TbHKs *in vitro* (Chambers, Kearns et al., 2008; Chambers, Fowler et al., 2008; Chambers, Morris et al., 2008; Albert et al., 2006) are appreciated, however, the control of expression and functional significance of these differing roles has remained largely unknown. Herein, we have demonstrated that the TbHK 3' UTRs can modulate differential expression of the two proteins in a stage dependent manner.

We have identified approximate regions in both TbHK1 and TbHK2 that contain positive and negative regulatory elements controlling expression. The identification of more than one regulatory element in the TbHK1 3'UTR suggests that regulation of this gene is complex and influenced by many different environmental triggers.

The observation that the 3' UTRs of both TbHKs harbor at least one copy of a K box and associated CAAC motif identified in other species (Lai et al., 1998; Lai, 2002; Muttray et al., 2005) is of particular interest. In these species, families of developmentally regulated genes are often coordinately controlled by these post-transcriptional regulatory elements (Fang and Qiu, 2009; Lai et al., 2005; Li et al., 2009). While we have not identified other members of a K-box regulated family in *T. brucei*, the stage dependent switch in metabolism and associated changes in protein expression profiles (especially of glycolytic enzymes) indicates a distinct possibility for such a

mechanism in the parasite. In addition, variability of K-box target sequence frequency as observed for the TbHKs occurs among other developmentally associated genes (Lai et al., 1998) and could confer variable sensitivity or expression patterns.

In our subsequent analysis of the functional capacity of these K-box elements, the proximal K-box of TbHK1 was revealed to confer a strong negative post-transcriptional regulatory function. The sensitivity of this element to a single nucleotide substitution suggests a stringent sequence requirement in agreement with the high conservation observed in other species (Lai et al., 1998; Muttray et al., 2005; Wu et al., 2008). mfold analysis of our mutant K-box reveals no structural changes further implying a *trans*-regulatory function. In contrast, the K-box of TbHK2 demonstrates little alteration of expression when similarly mutated. Under standard procyclic growth conditions, it appears as though the K-box of TbHK2 is either: 1) not a regulatory element, 2) not stringently adherent to sequence constraints, or 3) not actively involved in regulation.

In probing for elements which contain the seed sequence complimentary to the K-box target as would be expected of a *trans*- regulatory ncRNA, we have identified the first instance of an RNA transcribed from the anti-sense strand of the 3' UTR of a transcript for which it is capable of altering expression in trypanosomes. This is interesting as the transcript is produced from a DNA strand from which transcription was previously considered unlikely as such a mechanism would not conform to canonical, polycistronic transcription of the trypanosome genome. Whether this transcript represents a mature ncRNA or a precursor to some smaller RNA species (such as a miRNA) remains to be determined; however, the capacity to restore basal expression level when

co-ordinately introduced in super-cellular concentration with a mutant proximal TbHK1 K-box reporter is suggestive of a miRNA mechanism.

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CONCLUSION

Trypanosoma brucei, the causative agent of African sleeping sickness, expresses two hexokinases, TbHK1 and TbHK2, in both BSF and PF parasites (Colasante et al., 2006). The current treatments available for sleeping sickness are toxic and difficult to administer in underdeveloped countries (Brun et al., 2010). Hexokinase is considered a plausible drug target as glycolysis is the sole method used for ATP production in bloodstream form parasites. Deeper understandings of the regulation of gene expression and regulation of enzyme activity for this essential protein could therefore have a great impact on development of an effective therapeutic.

Regulation of Gene Expression

The study of gene expression regulation and how it occurs in *T. brucei* has long been a topic of interest due to the polycistronic organization of the genome, which results in a lack of transcriptional regulation (Haile and Papadopoulou, 2007). It is important for TbHK1 to be regulated properly, as previous studies reveal that misregulation of the enzyme are harmful to the cell (Albert et al., 2005; Chambers et al., 2008).

Through studying both the TbHK1 and TbHK2 3'UTRs and searching for elements involved in the regulation of gene expression, I have made several observations. First, regulation of gene expression is complex and likely involves a myriad of both *–cis* and *–trans* regulatory elements in order for gene expression to be executed properly. Initial studies to identify elements located in the hexokinase 3'UTRs involved in regulation revealed that there was no single element responsible for regulation, but instead this process involves several positive and negative elements that likely act in concert to regulate expression tightly. The notable length of

the TbHK1 3'UTR (1352 nt compared to the average of 604 nt) also supports this possibility (Siegel et al., 2010).

Second, the complexity of the regulation is likely closely linked to the unstable environmental conditions of the parasite. The complexity may allow the trypanosome to quickly and efficiently adapt its gene expression to subtle (in the microenvironment) and gross environmental changes (for example, the switch from mammalian host to insect vector). In order for the parasite occupy diverse niches, they must first be able to sense environmental changes effectively, and then be able to respond rapidly in order to survive. Previous studies to identify regulatory elements in the 3'UTRs of PF surface proteins reveal that nutrients play a role in regulating gene expression (Hehl et al., 1994). Similarly, I have shown that the hexokinases also harbor elements in their 3'UTRs that respond to carbon source availability. I have not yet identified the mechanism of this regulation and it likely entails regulation of both steady state transcript and protein translation levels. However, it is clear that the parasite does change gene expression in response to carbon source availability.

Lastly, my studies on differential polyadenylation reveal that some 3'UTR lengths are stabilized under certain environmental conditions; however, protein is not translated under those same environmental conditions. Though logically it seems transcribing mRNAs that are not needed right away may not be the most efficient use of the cell resources, further thought reveals that it may be a mechanism that the parasite employs in preparation for rapid changes. A recent review in Science by Keith Matthews states "For such a rapid-response capability (switch from host to vector), a common strategy is to hold pre-made mRNAs ready for translation, rather than relying on new gene transcription and then protein synthesis (Matthews, 2011)."

Future efforts from this work include the identification of the elements that bind the 3'UTRs, which may consist of nucleic acids and/or proteins. Further work will also involve exploring the genome for other similarly regulated genes. A high throughput analysis of whole genome expression of cells grown under different conditions could be utilized to identify such genes.

Regulation of Hexokinase Activity

Mechanisms of hexokinase activity are unique in *T. brucei*. Unlike hexokinases in other organisms TbHKs are not inhibited by their products (Nwagwa, 1982; Cronin, 1985). As aforementioned, the parasite must tightly regulate hexokinase gene expression subsequently, it must also regulate and maintain hexokinase activity under certain conditions. Here I report that Gly3P is capable to regulate TbHK activity in an acidic environment. The discovery of this means of regulation implies that the parasite utilizes small molecules that are readily available in the cell to maintain the catalytic activity of this important metabolic enzyme. This regulatory mechanism may play an important role in the maintenance of stable metabolism as the parasite traverses through its life cycle. Similar to rapid responses in gene regulation, enzyme activity must also remain tightly regulated to allow the parasite to effectively generate ATP as carbon source availability and cellular conditions change.

Hexokinase as a Drug Target

Lastly, I have been involved on studies further confirming hexokinase as a viable drug target for the *T. brucei*. As previously mentioned, current treatments available for trypanosomiasis are somewhat toxic and hard to administer. Hexokinase is considered a

plausible drug target because it is essential to BSF parasites and also due to its low similarity to human glucokinase. I have been involved in characterization of quercetin, a previously recognized trypanocide, as a mixed inhibitor of TbHK1. Through the study of quercetin a more clear understanding of hexokinase binding and enzyme conformation has been revelated. Currently, studies are underway in our lab and in collaboration with other labs to identify potent and effective inhibitors of hexokinase that could potentially be optimized and developed into a therapeutic. Overall, the far reaching consequences and detriment of African sleeping sickness reveals the continued importance of studying the basic biology of the parasite to in turn be able to develop an effective treatment for the disease.

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APPENDIX

Appendix A

A TARGET-BASED HIGH THROUGHPUT SCREEN YIELDS *TRYPANOSOMA BRUCEI* HEXOKINASE SMALL MOLECULE INHIBITORS WITH ANTIPARASITIC ACTIVITY

Elizabeth R. Sharlow[1,2], Todd A. Lyda[3], Heidi C. Dodson[3], Gabriela Mustata[4], Meredith T. Morris[3], Stephanie S. Leimgruber[1,2], Kuo-Hsiung Lee[5], Yoshiki Kashiwada[6], David Close[1,2] John S. Lazo[1,2], and James C. Morris[3#]

From the [1]University of Pittsburgh Drug Discovery Institute and Pittsburgh Molecular Libraries Screening Center, University of Pittsburgh, Pittsburgh, PA, USA, the [2]Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA USA, the [3]Department of Genetics and Biochemistry, Clemson University, Clemson, SC, USA, and the [4]Department of Computational Biology, University of Pittsburgh, Pittsburgh, PA, USA

[5]Natural Products Research Laboratories, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA

[6]Graduate School of Pharmaceutical Sciences, University of Tokushima, Tokushima, Japan

ABSTRACT

The parasitic protozoan *Trypanosoma brucei* utilizes glycolysis exclusively for ATP production during infection of the mammalian host. The first step in this metabolic pathway is mediated by hexokinase (TbHK), an enzyme essential to the parasite that transfers the γ-phospho of ATP to a hexose. Here we describe the identification and confirmation of novel small molecule inhibitors of bacterially expressed TbHK1, one of two TbHKs expressed by *T. brucei,* using a high throughput screening assay.

Methodology/Principal Findings: Exploiting optimized high throughput screening assay procedures, we interrogated 220,233 unique compounds and identified 239 active compounds from which ten small molecules were further characterized. Computation chemical cluster analyses indicated that six compounds were structurally related while the remaining four compounds were classified as unrelated or singletons. All ten compounds were ~20-17,000-fold more potent than lonidamine, a previously identified TbHK1 inhibitor. Seven compounds inhibited *T. brucei* blood stage form parasite growth ($0.03 \leq EC_{50} < 3$ μM) with parasite specificity of the compounds being demonstrated using insect stage *T. brucei* parasites, *Leishmania* promastigotes, and mammalian cell lines. Analysis of two structurally related compounds, ebselen and SID 17387000, revealed that both were mixed inhibitors of TbHK1 with respect to ATP. Additionally, both compounds inhibited parasite lysate-derived HK activity. None of the compounds displayed structural similarity to known hexokinase inhibitors or human African trypanosomiasis therapeutics.

Conclusions/Significance: The novel chemotypes identified here could represent leads for future therapeutic development against the African trypanosome.

Author Summary

African sleeping sickness is a disease found in sub-Saharan African that is caused by the single-celled parasite *Trypanosoma brucei*. The drugs used widely now to treat infections are 50 years old and notable for their toxicity, emphasizing the need for development of new therapeutics. In the search for potential drug targets, researchers typically focus on enzymes or proteins that are essential to the survival of the infectious agent while being distinct enough from the host to avoid accidental targeting of the host enzyme. This work describes our research on one such trypanosome enzyme, hexokinase, which is a protein that the parasite requires to make energy. Here we describe the results of our search for inhibitors of the parasite enzyme. By screening 220,223 compounds for anti-hexokinase activity, we have identified new inhibitors of the parasite enzyme. Some of these are toxic to trypanosomes while having no effect on mammalian cells, suggesting that they may hold promise for the development of new anti-parasitic compounds.

INTRODUCTION

African sleeping sickness conjures historical images of disease-induced fatal slumbering striking down men, women, and children, consequently decimating villages of colonial Africa. Unfortunately, people living in many countries of sub-Saharan Africa today know that African sleeping sickness is *not* a disease of history but rather is a much-neglected disease of the present, particularly in areas that suffer the additional burdens of war, famine, global and local climate changes, and other infectious agents. The causative agents of sleeping sickness (or human African trypanosomiasis, HAT) are subspecies of the African trypanosome *Trypanosoma brucei.*

Approximately 500,000 people in sub-Saharan Africa are infected annually with the parasite leading to 50,000-70,000 deaths per year [1]. Similar to other neglected tropical diseases, limited therapeutics for HAT are available and of the drugs currently used, most have serious adverse side effects, including encephalopathy, toxicity, and death [2]. Thus, there is a desperate need for new HAT therapeutics with the preference shifting from general cytotoxic agents towards molecular target-based therapeutics that should display fewer toxic effects.

Bloodstream form (BSF) *T. brucei* parasites generate ATP exclusively through glycolysis and *T. brucei* hexokinase TbHK, the first enzyme in glycolysis, has previously been validated as a target for therapeutic development. In these experiments, BSF parasites were shown to be sensitive to RNA interference (RNAi)-based silencing of TbHKs [3,4], with cell toxicity observed after 3-5 days of RNAi exposure. Additonally, known inhibitors of HKs have been demonstrated to inhibit *T. brucei* hexokinase 1 (TbHK1), one of two nearly identical TbHKs that the parasite expresses. These compounds are furthermore toxic to the parasite [4]. While some mammalian HK inhibitors can inhibit TbHK1, TbHK1 is distinct enough from mammalian HKs to suggest that it can be specifically targeted. Supporting this notion, TbHK1 shares only 30-33% sequence identity with the mammalian HKs and differs further by unusual oligomerization into hexamers [5]. Moreover, the unusual spectrum of known inhibitors of the trypanosome enzymes, including fatty acids and other small molecules (like pyrophosphate, [5]), support the idea that this essential parasite protein is sufficiently distinct from any mammalian counterpart to make an ideal target for therapeutic development. Indeed, targeting TbHK using structurally based inhibitors has yielded trypanocidal compounds, albeit at high concentrations [6,7].

Here we describe our high throughput target-based approach to identify specific inhibitors of the essential parasite enzyme, TbHK1. Overall, ten compounds were confirmed as

novel TbHK1 small molecule inhibitors exhibiting little or no similarity to known HK inhibitors (or HAT therapeutics). Most of the potent TbHK1 inhibitors were toxic to culture-grown BSF *T. brucei* while not exhibiting toxicity towards mammalian cells, suggesting that they may be useful lead compounds in the development of new therapies for African trypanosomiasis.

METHODS

Chemicals and Reagents. Clear 384-well microtiter plates were purchased from Greiner (Monroe, NC) and used for all experiments. Glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide (NAD^+), adenosine triphosphate (ATP), lipoic acid (PubChem SID 11532893) and glucose were purchased from Sigma (St. Louis, MO). Phosphoenol pyruvate (PEP), ebselen (PubChem SID 856002) and glucosamine were obtained through VWR (West Chester, PA) and dimethyl sulfoxide (DMSO) was purchased from Fisher (Pittsburgh, PA). The following PubChem SID compounds were obtained from commercial vendors: 3716597, 24830882, 17386310, and 16952891 (Enamine/Kiev, Ukraine); 24797131 (Chembridge/San Diego, CA); 14728414 and 17387000 (Specs/Delft, The Netherlands); 17507245 (Asinex/Moscow, Russia); and 24785302 (ChemDiv, San Diego, CA).

Compound Libraries. The library of pharmacologically active compounds (LOPAC) (1,280 compounds) was purchased from Sigma-Aldrich. The Pittsburgh Molecular Libraries Screening Center (PMLSC) provided the 220,233 compound library screened for TbHK1 small molecule inhibitors, which was made available as part of the NIH Molecular Libraries Roadmap Initiative. Cherry-picked compounds from the PMLSC library were supplied by BiofocusDPI (San Francisco, CA).

Purification of Bacterially Expressed TbHK1. For purification of bacterially expressed TbHK1 (rTbHK1), a previously described protocol [8] was modified to increase yield. Briefly, a starter culture of *E. coli* M15(pREP) harboring pQE30 (Qiagen, Valencia, CA) with the TbHK1 gene cloned in frame of a 6-His tagging sequence was grown in ECPM1 [9] and then inoculated into a 5 L bioreactor (Biostat B, B. Braun Biotech International, Allentown, PA) and grown at 37^{o}C. At OD_{600} between 3-5, the culture was induced with IPTG (0.8 mM), grown without supplement O_2 (37^{o}C, 16 hr), and cells collected by centrifugation (5000 x g, 20 min, 4^{o}C). The pellet was resuspended in lysis buffer (50 mM $NaPO_4$, pH 8.1, 5 mM glucose, 150 mM NaCl, and 0.1% Tween) and lysed by using a cell disruptor (Constant Cell Disruption Systems, Sanford, NC). The resulting supernatant was applied (5 ml/min) to a 50 ml ProBind column (Invitrogen, Eugene, OR) on a FPLC (GE Lifesciences, Piscataway, NJ) and protein eluted by gradient (5 to 250 mM imidazole) in lysis buffer. Fractions were screened using HK activity assays and Western blotting and those containing rTbHK1 were pooled, concentrated, and applied to a HiTrap SP HP column (GE Lifesciences, Piscataway, NJ).

Automated primary TbHK1 HTS and glucose-6-phosphate dehydrogenase coupled assays. TbHK1 assays were an adaptation of a coupled enzyme HK assay to a 384-well format [8,10]. Briefly, test and control compounds (30 μM in 15 μL volume) were added to a 384 well black, opaque microtiter plate using a Velocity 11 V-prep (Santa Clara, CA) for a final test compound concentration of 10 μM. Negative (vehicle) controls contained 1% DMSO, positive controls contained 133 mM glucosamine and IC_{50} controls contained 1.3 mM glucosamine (final well concentrations). A mixture containing glucose (1.5 mM), ATP (1.05 mM), $MgCl_2$ (4.5 mM),

NAD^+ (9 mM), glucose-6-phosphate dehydrogenase (G6PDH, 0.018 mUnits/µL) and triethanolamine (TEA, 100 mM, pH 8.0) in a 15 µL volume was then added to each well of the assay plate using a Perkin Elmer FlexDrop (Waltham, MA) followed by addition of rTbHK1 (1.5 ng/µl in 15µL volume). The 45 µL reaction mixture was incubated at RT for 2 hr and then quenched with 5 µL EDTA (500 mM). The resulting signal, which remained stable for up to 5 hr after addition of stop reagent, was collected on a Molecular Devices SpectraMax M5 (absorbance at OD_{340})(Sunnyvale, CA).

To account for possible inhibition of the reporter enzyme in the primary coupled reaction, putative inhibitors were screened to assess their activity against a G6PDH coupled assay. Briefly, test and controls compounds were added to the wells of a 384 well assay plates as described above. Negative (vehicle) controls contained 1% DMSO, positive controls contained 100 mM PEP and IC_{50} controls contained 8.6 mM PEP (final well concentrations). A mixture containing glucose-6-phosphate (G6P, 0.6 mM) and NAD^+ (1.8 mM) in a volume of 15 µL was then added to each assay plate well. The reaction was initiated by addition of 15 µL G6PDH (0.018 mUnits/mL) (for a final volume of 45 µl), incubated at RT for 1 hr, and then quenched with 5 µl of EDTA (500 mM). The change in absorbance at OD_{340} was monitored as above.

Additional specificity assays were performed using human HK 4 (human glucokinase, hGlk, GenBank accession no. BC001890) that was expressed from a cloned cDNA (OPEN Biosystems, Huntsville, AL) in pQE30. After sequencing, the plasmid was transformed into *E. coli* M15 (pREP) and cultures were grown to an OD_{600} of 0.9 in terrific broth and protein expression induced (3 hr, 37°C) with 1 mM IPTG followed by purification by nickel-affinity chromatography.

Inhibition assays of lysate-derived TbHK. Parasite lysates from BSF parasites were prepared by incubation (5 min on ice) of 1.5 x 10^7 cells in lysis buffer (0.1 M TEA, pH 7.4, and 0.1% Triton X-100) supplemented with 1 mM PMSF, 5 μg/ml leupeptin, and 100 μg/ml TLCK. In triplicate, cell equivalents (2 x 10^5) were incubated with increasing concentrations of inhibitor for 15 minutes at RT prior to initiation of the coupled reaction. In short, the 200 μl reactions included 50 mM TEA, pH 7.4, 33 mM $MgCl_2$, 20 mM glucose, 5.25 mM ATP, 0.75 mM NADP, and 0.1 units of G6PDH, with kinetic analyses performed using KaleidaGraph 4.1 (Synergy Software, Reading, PA).

T. brucei viability assay. To determine the impact of TbHK1 inhibitors on cell growth, we seeded 5 x 10^3 BSF parasites (cell line 90-13, a 427 strain) into 96-well clear-bottomed polystyrene plates in 200 μl HMI-9 supplemented with 10% fetal bovine serum and 10% Serum Plus (Sigma-Aldrich, St. Louis, MO) and grown in the presence of compound (2 μl) or equivalently diluted carrier for 3 days in 5% CO_2 at 37^{o}C. CellTiter Blue (Promega, Madison WI) was added (20 μl) and the plates incubated an additional 3 hr under standard culture conditions. Fluorescence emission at 585 nm was then measured after excitation at 546 nm in a GENios microtiter plate reader (Phenix Research Products, Hayward CA). DMSO solvent was maintained at or below 1%, with 1% causing a 16% reduction in cell number at the end of the three day assay.

Procyclic form (PF) parasites (29-13, a 427 strain, 5 x 10^4/well) were grown in 96-well clear-bottomed polystyrene plates in 200 μl SDM-79 for 2 days (5% CO_2, 25^{o}C) and then CellTiter Blue (20 μl) added. Plates were then incubated for 1 hr under standard culture conditions. Fluorescence of samples was then characterized as above.

Mammalian cell-line and Leishmania promastigote specificity assays. Cell-based specificity assays were performed as previously described [11]. Briefly, mammalian cell line and *Leishmania* promastigote assays were performed in final volumes of 25 µL using our previously described 384-well microtiter format [12]. All mammalian cell lines were cultured and maintained in complete growth medium preparations according to ATCC specifications (ATCC, Manassas, VA). *Leishmania* promastigotes were cultured as previously described [11]. A549 (1,000 cells/22 µL), IMR-90 (1,000 cells/22 µL), HeLa (1,000 cells/22 µL), MDA-MB-231 (3,000 cells/22 µL), *Leishmania* promastigotes (5,000 parasites/22 µL) were seeded into each well of 384-well microtiter plates and test and control compounds (3 µl) were added to individual wells. Vehicle and positive controls were 1% and 10% DMSO, respectively. For mammalian cells, assay plates were incubated for 44-46 h at 37^{o}C in the presence of 5% CO_2 and for the *Leishmania* promastigotes, assay plates were incubated for 44 h at 28^{o}C with 5% CO_2. Five µL of CellTiter Blue reagent was added to each assay plate well and incubated for 2-4 h at 37^{o}C with 5% CO_2. Data were captured on a Molecular Devices SpectraMax M5 (excitation A_{560}; emission A_{590}).

HTS data analysis and statistical analysis. Primary HTS data analysis and subsequent compound IC_{50} calculations were performed using ActivityBase (IDBS, Guilford, UK) and Cytominer (University of Pittsburgh Drug Discovery Institute, Pittsburgh, PA). Structural similarity of the confirmed inhibitors was determined using Leadscope software (Columbus, OH). Additional visualization and statistical analysis were performed using GraphPad Prism software 5.0 and Spotfire (Somerville, MA). The PubChem database

(http://PubChem.ncbi.nim.nih.gov) was used to verify if the confirmed TbHK1 small molecule inhibitors exhibited bioactivity in other assays.

***In silico* ADME/Toxicity analysis.** Computational modeling tools were used to estimate the bioavailability, aqueous solubility, blood brain barrier potential, human intestinal absorption, the cytochrome P450 (*i.e.* CYP2D6) enzyme inhibition potential, mutagenicity, and hERG inhibition of the confirmed TbHK1 inhibitors. The bioavailability, aqueous solubility, and human intestinal absorption were estimated using the ADME Boxes v4.0 software (Pharma Algorithms, Toronto, Canada), while mutagenicity and hERG inhibition were estimated with TOX Boxes v2.9 software (Pharma Algorithms, Toronto, Canada). The CYP2D6 inhibition and blood brain barrier potential were predicted using Accord for Excel 6.2.2 (Accelrys, Inc, San Diego).

RESULTS

Validation of optimized HTS assay conditions using the LOPAC set. The TbHK1 coupled assay was optimized and validated for HTS by screening the LOPAC set. Compounds were assayed in duplicate at a single concentration (10 μM) and reproducibility between the duplicate screens is represented in Fig. 1 (R^2= 0.96). Average Z-factors were 0.69 ± 0.02 for the two LOPAC assays demonstrating the robustness of the developed assay format [13]. Eighteen compounds inhibited TbHK1 enzymatic activity ≥ 40% at 10 μM including myricetin, a structural analog of quercetin, which was previously identified as a TbHK1 small molecule inhibitor (IC_{50} = ~85 μM) (Lyda and Morris, unpublished). These data confirmed that our automated HTS assay conditions were robust and could be used to identify compounds that inhibited TbHK1 activity.

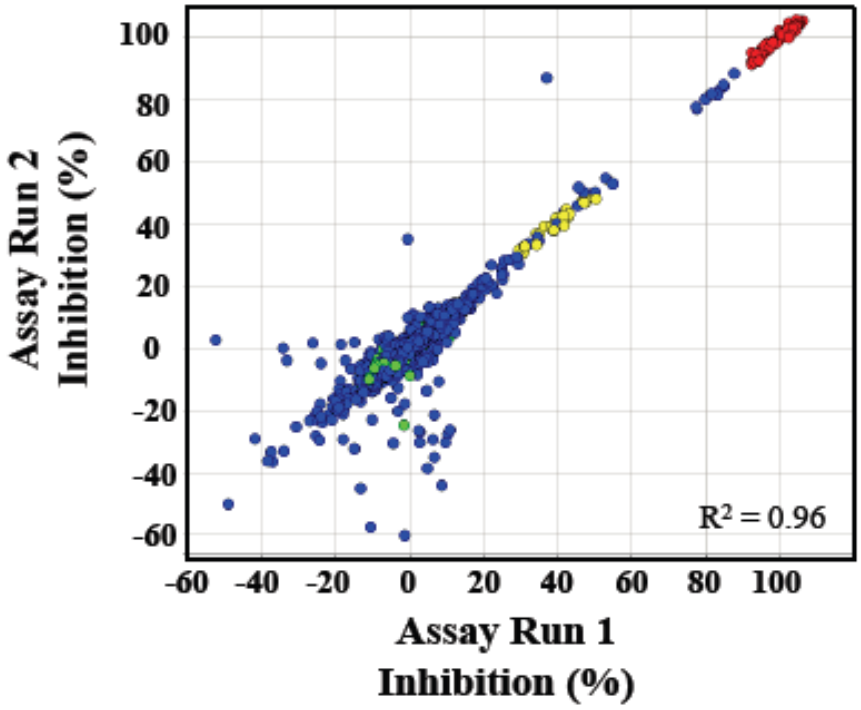

Figure A.1. Validation of the HTS by LOPAC screening.

Plot of percent inhibition for duplicate screen of the 1280 LOPAC compounds. LOPAC compounds (blue), minimum control, which should equal ~100% inhibition of signal readout (red), IC_{50} control compounds (yellow), and maximum control compounds, which should equal ~0% inhibition of signal readout (green), are indicated.

Interrogation of 220,233 compounds for TbHK1 small molecule inhibitors. We next screened 220,223 compounds at a single concentration (10 μM) for small molecule inhibitors of TbHK1 (Fig. 2). The HTS assay performed robustly (average Z-factors of 0.80 ± 0.1) and identified 239 compounds as primary actives (>50% inhibition at 10 μM), for an overall hit rate of 0.1%. The 239 active compounds were cherry-picked, and the initial inhibitory activity confirmed in the primary TbHK1 assay. Additionally, the compounds were tested against the reporter enzyme,

G6PDH, to confirm that they did not interfere with the assay format. Following initial 20 point IC_{50} value determinations using cherrypicked compounds, compounds with IC_{50} values < 50 μM were obtained from commercial sources. The activity of the 13 resupplied compounds was empirically determined to control for possible TbHK1 inhibitory effects associated with compound library degradation. Ten small molecules confirmed with TbHK1 IC_{50} values < 50 μM while three compounds failed to inhibit TbHK1. Leadscope analysis of the 10 confirmed TbHK1 inhibitors classified six compounds into a cluster of structurally related compounds (cluster 1) while the remaining four compounds were classified as singletons (Table 1, Fig. 3). Ebselen (SID 856002) was the most potent compound in cluster 1 with an IC_{50}=0.05 ± 0.03 μM. For the majority of the compounds IC_{50} values either improved or remained similar to cherry-picked compounds with the exceptions of SID 17386310 and SID 14728414 (Table 1) which were 7.5 and 6.6-fold less potent, respectively, upon resupply (data not shown). Moreover, all ten novel TbHK1 inhibitors were 20-17,000-fold more potent than londiamine, a previously described TbHK1 inhibitor [4] and 2-1720-fold more potent than quercetin (Lyda and Morris, unpublished).

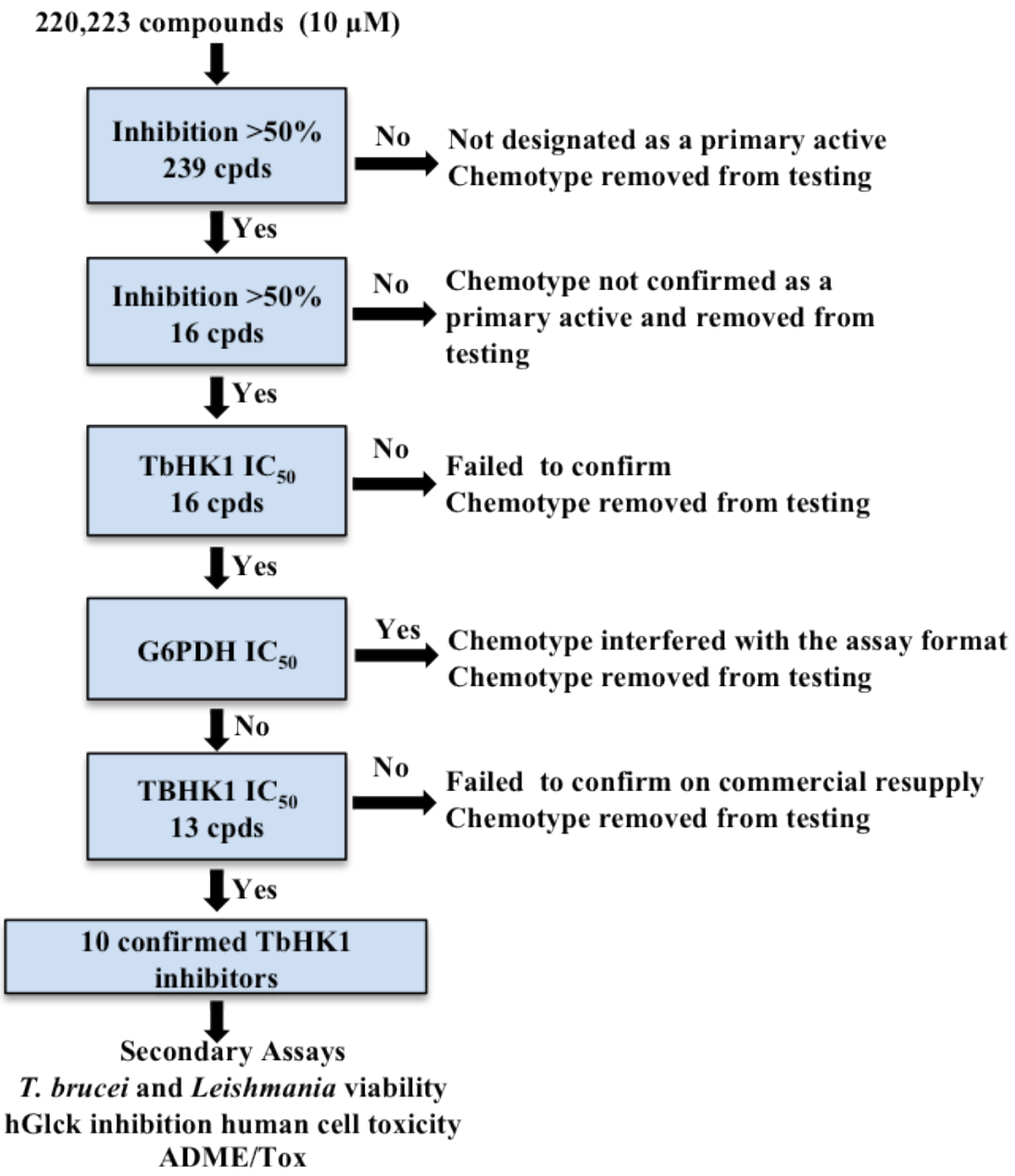

Figure A.2. Scheme depicting HTS interrogation of a 220,233 small molecule library for TbHK1 inhibitors.

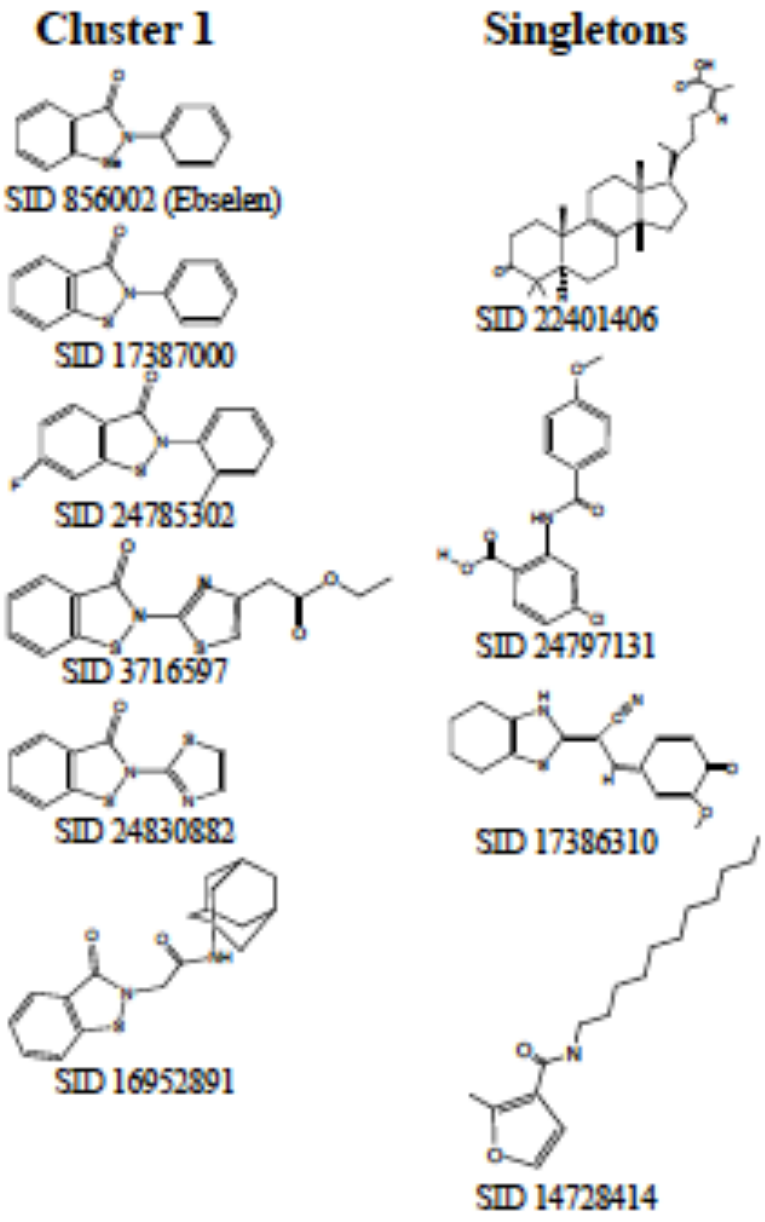

Figure A.3. Structures of HTS Cluster 1 and Singleton Hits

Additional experiments to assess the *in vitro* specificity included testing the compounds against hGlk. The activity against hGlck was varied, with the cluster 1 compounds yielding a spectrum of efficacy, from very low inhibition at 10 μM (for example, SID 17387000, with 6.7% inhibition, Table 1) to near complete inhibition by ebselen (97.8% inhibition). Singletons also demonstrated a spectrum of activity against hGlck with SID 22401406 and SID 14728414 having minimal impact on the enzyme while 17386310 was a more potent inhibitor (Table 1). Moreover, data mining of the PubChem database (http://pubchem.ncbi.nlm.nih.gov/) determined the frequency with which a compound was found to be active in other assays. In general, the cluster 1 compounds were active in other assays more frequently than the singletons, with SID 17387000 the most frequently active (identified in 7.2% of the 238 assays in which it was tested). Singletons, on the other hand, were less frequently active. For example, SID 14728414 was confirmed as an active in one of 216 assays (<1%) (Table 1).

TbHK1 small molecule inhibitors are toxic to BSF parasites. TbHK1 has previously been shown to be an essential gene for BSF *T. brucei* [4], suggesting that inhibitors of the enzyme may be promising lead compounds for therapeutic development. To initially explore this possibility, we grew cultured BSF parasites in the presence of 10 μM compound and cell density monitored after 72 hr (Table 1). The ten resupplied compounds were tested in this assay, with EC_{50}s determined for those that inhibited cell growth >50% at 10 μM (Table 1). Compounds in cluster 1 included two of the most potent anti-trypanosomal compounds, SID 17387000 and SID 24785302. Singletons were also toxic, with SID 17386310 being one of the most potent compounds tested to date. Four molecules, including a member of cluster 1 (SID 3716597) and three singletons (SID 22401406, SID 24797131, SID 14728414) inhibited TbHK1 but were not

toxic to BSF at 10 μM. With the exception of ebselen, the resupplied anti-parasitic compounds exhibited EC_{50} values that were 10-1000 fold lower than the TbHK1 IC_{50} values. This discrepancy could result from other actions or concentration of the inhibitors in the glycosome, which is a small peroxisome-like organelle where TbHK1 is located. Additionally, we have found that RNAi of TbHK1, which reduces expression but does not necessarily ablate it, is toxic to BSF parasites, suggesting that modest inhibition of cellular TbHK1 activity could be lethal to the parasite [4].

To explore the likelihood of off-target whole parasite effects, we assessed the toxicity of the TbHK1 inhibitors against PF parasites. Unlike BSF parasites, PF parasites can utilize both amino acids and glucose for ATP production. This dynamic metabolism suggests that the PF parasites may be less sensitive to TbHK1 inhibitors. Indeed, at 10 μM most of the resupplied compounds had only a modest impact on PF parasite growth, inhibiting growth between 0-51% when compared to control cell lines. Compounds toxic to *T. brucei* were also assayed against a related kinetoplastid parasite, *Leishmania*. The *Leishmania* promastigotes were typically less sensitive to the resupplied compounds (with EC_{50}s >12.5 μM), with the exception of the cluster 1 compounds ebselen, SID 17387000, SID 24785302 and the singleton, SID 17386310. These compounds had EC_{50} values against *Leishmania* between 1-5 μM in exponentially growing parasites (Table 1). Moreover, our identified TbHK1 inhibitors have minimal impact on human cell lines with EC_{50} values >12.5 μM, suggesting at least 400-fold greater toxicity toward parasites for the most potent *T. brucei* cytotoxic compounds (Table A.1).

Table A.1. HTS Cluster 1 and Singleton Hits

Leadscope Grouping	PubChem SID	PubChem Bioassay Activity[1]	IC_{50} (μM) Resupply	% Inhibition of hGlck (10 μM)	BSF EC_{50} (μM)	% PF Growth Inhibition (10 μM)	L. major EC_{50} (μM)
Cluster 1	856002 (Ebselen)	344/44/22	0.05 ± 0.03	97.8 ± 0.1	2.9 ± 0.28	51 ± 0.16	4.1 ± 0.4
	17387000	236/38/17	2.0 ± 0.5	6.7 ± 9.4	0.030 ± 0.067	48 ± 0.15	1.9 ± 0.2
	24785302	170/21/11	4.2 ± 1.0	6.9 ± 4.2	0.042 ± 0.0028	47 ± 0.15	1.9 ± 0.2
	3716597	318/29/18	9.3 ± 0.3	7.8 ± 7.1	> 10	27 ± 0.080	>12.5
	24830882	171/12/7	16.9 ± 0.1	88.8 ± 4.9	0.83 ± 0.20	8.6 ± 0.030	>12.5
	16952891	214/24/11	2.6 ± 0.2	44.9 ± 9.9	0.30 ± 0.079	47 ± 0.15	>12.5
Singletons	22401406	184/5/2	2.3 ± 0.3	0.0	> 10	0.0	>12.5
	24797131	175/6/2	11.4 ± 3.2	6.3 ± 8.8	> 10	0.0	>12.5
	17386310	211/10/4	33.6 ± 10.2	70.3 ± 3.6	0.038 ± 0.0038	50 ± 0.15	2.6 ± 0.1
	14728414	216/2/1	41.7 ± 3.0	1.9 ± 7.0	> 10	0.0	>12.5

[1]As of 09/03/09. Number of bioassays in which the compound was tested/number in which the compound was active/number in which the compound has been confirmed as an inhibitor.

In silico predictions for the identified TbHK1 inhibitors. To investigate the chemical similarity of our newly identified TbHK1 small molecule inhibitors to current treatments for African sleeping sickness as well as previously described TbHK1 inhibitors, we performed a similarity search using the Tanimoto coefficient. The data indicated that the TbHK1 inhibitors displayed low levels of similarity with all compounds examined with the highest similarity being between lonidamine and SID 16952891 (47%) (Table A.2). Thus, these results demonstrate that the newly identified TbHK1 inhibitors are unique, either displaying no or very low similarity to known TbHK1 inhibitors and current therapies for African sleeping sickness.

Table A.2. Comparison of structural similarities of HTS hits to licensed compounds used against HAT and to known TbHK1 inhibitors

Compound	Proposed Mode of Antiparasitic Action[1]	TbHK1 Inhibitor (SID)	Similarity Coefficient[2](%)
Pentamidine	Accumulation in the mitochondria, DNA binding	17386310	31
Suramin	Inhibition of glycolysis	24830882	38
		24797131	38
Melarsoprol	Inhibition of glycolysis Interaction with thiols	24830882	43
Eflornithine	Polyamine biosynthesis (via inhibition of ODC)	14728414	27
Lonidamine	TbHK1 inhibitor[3]	16952891	47
Quercetin	TbHK1 inhibitor[4]	24797131	38

[1]Reviewed in (13).
[2]Similarity coefficient was determined using the Tanimoto coefficient. Compounds with values greater than 80% are considered highly structurally similar.
[3](4)
[4]Lyda and Morris, unpublished

Additional *in silico* ADME-tox predictions indicated that all ten TbHK1 compounds had an extremely low probability for being either a hERG channel inhibitor or mutagenic (data not shown). Moreover, all compounds, except SID 3716597 were predicted to be moderately to highly bioavailable and nine of 10 compounds displayed medium to very high blood brain barrier (BBB) potential, with the majority of the cluster one compounds predicted to have high to very high BBB potential (Table A.3). Six of 10 TbHK1 inhibitors were predicted to have no inhibitory activity on CYP2D6 enzyme with the exceptions being cluster one compounds SID 3716597 and SID 24785301 and singletons SID 14728414 and SID 24797131 (Table 3). The majority of the compounds had a predicted low aqueous solubility (Table 3), suggesting that if

these compounds were to be used in future analogue development, they would need to be refined to improve their aqueous solubility. Thus, based on empirically derived data and *in silico* analyses, we focused on cluster 1 compounds for subsequent analyses.

Table A. 3. *In silico* ADME/Toxicity Analysis

Leadscope Grouping	PubChem SID	Aqueous Solubility	Bioavailability	CYP2D6 inhibition	Blood brain barrier
Cluster 1	856002 (Ebselen)	Low	High	Non-inhibitor	High
	17387000	Low	High	Non-inhibitor	High
	24785302	Low	Moderate	Inhibitor	Very High
	3716597	Low	Low	Inhibitor	Medium
	24830882	Good	High	Non-inhibitor	High
	16952891	Low	Moderate	Non-inhibitor	Medium
Singletons	22401406	Extremely Low	Moderate	Non-inhibitor	Undefined
	24797131	Good	High	Inhibitor	Medium
	17386310	Good	Moderate	Non-inhibitor	Medium
	14728414	Low	Moderate	Inhibitor	High

Further characterization of two structurally related cluster 1 TbHK1 small molecule inhibitors. Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one, was the most potent TbHK1 inhibitor (IC_{50} = 0.05 ± 0.03 μM) identified in our studies while the structurally related inhibitor SID 17387000 (2-phenyl-1,2-benzisothiazol-3(2*H*)-one) was the next potent compound identified with an IC_{50} = 2.0 ± 0.5 μM (Table 1). Analysis of the nature of TbHK1 inhibition revealed that both ebselen and SID 17387000 were mixed inhibitors with respect to ATP, with K_i values of 6.13 μM and 6.89 μM, respectively (Fig. 4). However, both ebselen and SID 17387000 exhibited comparable IC_{50} values against *T. brucei* lysate-derived TbHK1 enzymatic activity with IC_{50} values of 0.43 ± 0.02 μM and 1.2 ± 0.12 μM respectively (Fig. 5). Thus, while compound SID 17387000 was nearly as potent against lysate activity as the bacterially expressed protein, ebselen was significantly less potent against parasite lysate-derived TbHK1 activity (than the bacterially expressed protein). These results suggest that ebselen may be metabolized

by cellular components or that lysate-derived TbHK1 may be associated with various cofactors that result in less potent IC_{50} values. Ebselen was also a potent inhibitor of hGlck (97.8% inhibition at 10 μM), while SID 17387000 had relatively little activity (6.7% inhibition) against the kinase (Table 1). Moreover, ebselen was ~100-fold less active against BSF parasites (Table 1). Taken together, these data suggest that the subtle structural differences between the two compounds result in remarkable changes in their pharmacological behavior.

Both ebselen and SID 17387000 are potent trypanocides, with EC_{50}s of 2.9 ± 0.28 μM and 0.030 ± 0.067 μM, respectively. To determine if indeed the toxicity to BSF parasites is related to inhibition of cellular TbHK1, we measured the impact of the compounds on cellular G6P levels after culturing the parasites in the presence of ebselen and SID 17387000 [14] (Fig. 5C). To reduce the likelihood that toxicity was impacting G6P levels non-specifically, we limited the incubation period to 1 and 4 hours, while employing high doses (10 times the EC_{50}) of the compounds. Incubation with ebselen for either 1 or 4 hours led to a 56% or 70% reduction in G6P, while SID 17387000 was less effective, reducing G6P levels 29% and 18% after 1 and 4 hours, respectively (Fig. 5C). While these observations suggest a direct impact on TbHK activity, other off-target impacts could be ultimately responsible for toxicity.

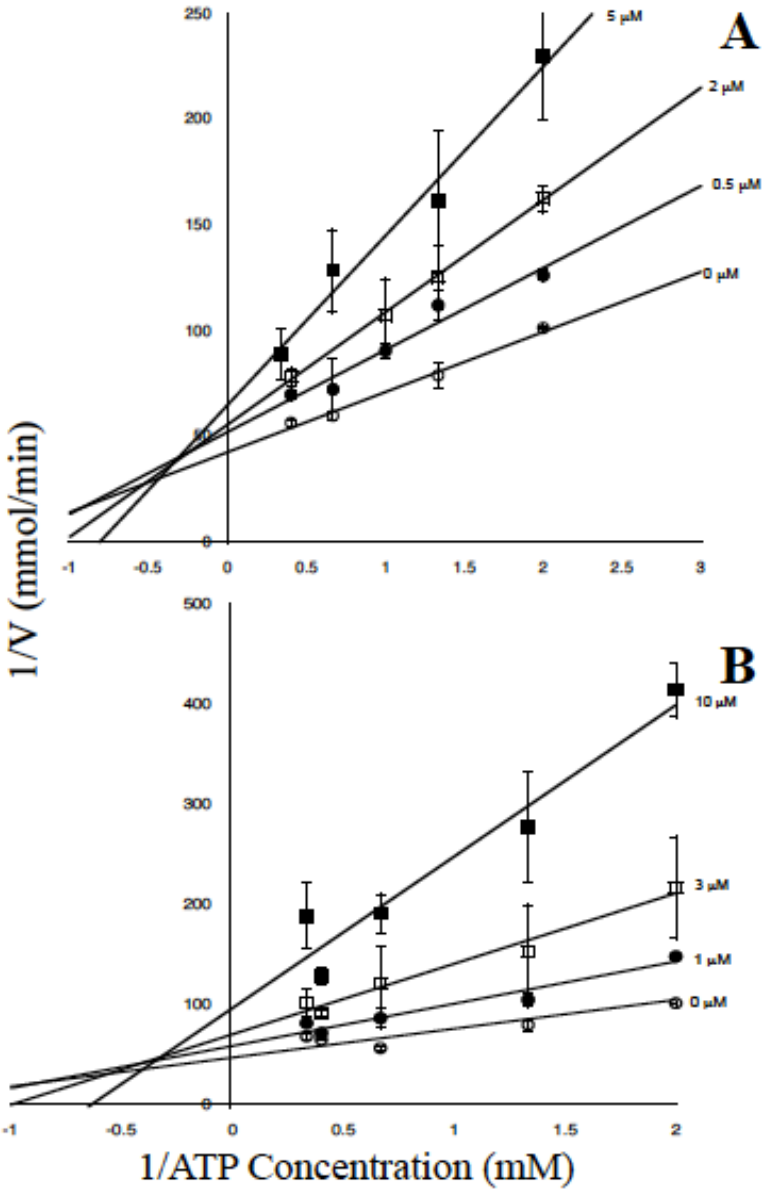

Figure A.4. Ebselen and SID 17387000 are mixed inhibitors of TbHK1 with respect to ATP. Lineweaver-Burk plots of inhibition with ebselen (A.) or SID 17387000 (B.). Assays were performed as described for cell lystates (see Materials and Methods) with ATP concentrations varied.

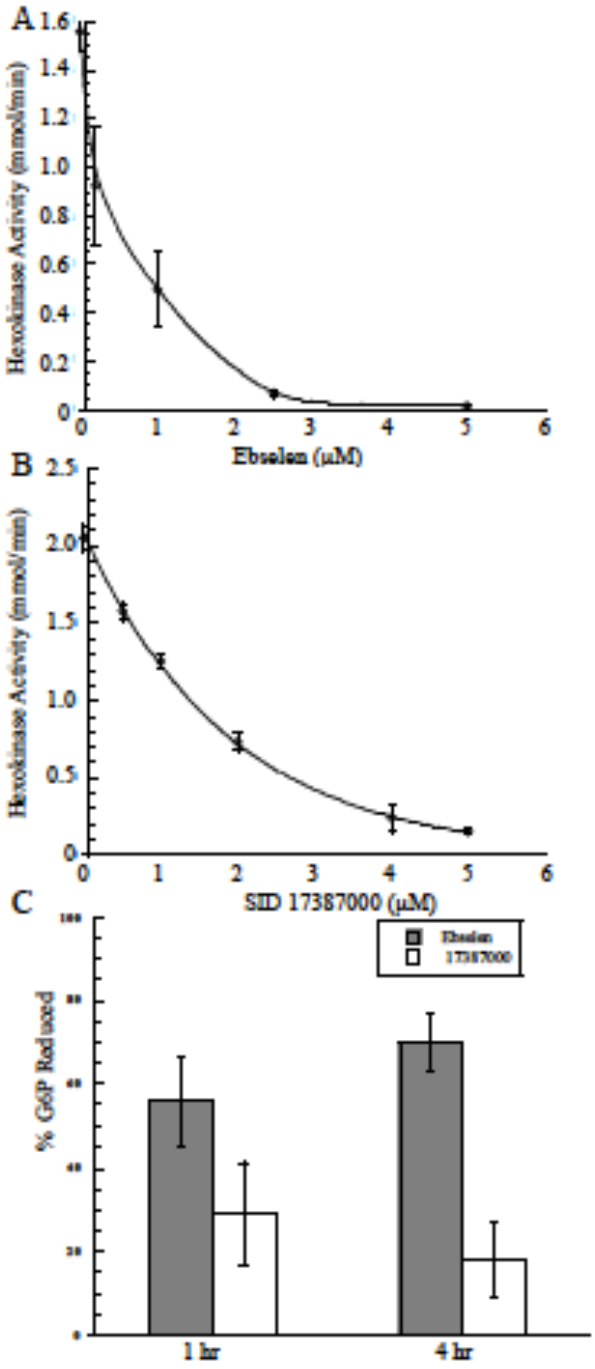

Figure A.5. Ebselen and SID 17387000 inhibit TbHK activity from parasite cell lysate and cause a reduction in cellular G6P levels in BSF parasites.

Increasing amounts of (A.) ebselen or (B.) SID 17387000 were incubated with 2 x 10^5 BSF cell equivalents for 15 min at RT and HK assays were performed as described in the Materials and Methods. (C.) Growth in the presence of Ebselen or SID 17387000 causes a reduction in cellular G6P levels. BSF parasites (1 x 10^7) were cultured for 1 or 4 hours in the presence of 30 μM or 1 μM (10-fold the EC_{50}) ebselen or SID 17387000 followed by lysate preparation and comparison of G6P levels to an equivalent number of untreated parasites [14].

DISCUSSION

There are currently four drugs approved for treatment of HAT. However, suramin and pentamidine, developed in 1921 and 1941, respectively, are not effective against the late stage of disease that occurs when the parasite crosses the blood-brain barrier. Melarsoprol, which was introduced in 1949, leads to fatal complications in 5-10% of patients receiving the drug [15]. The most recently developed drug, eflornithine, is only efficacious against *T. b. gambiense*, but is curative for both the early blood-borne infection and the late stage of disease with central nervous system involvement; delivery of eflornithine is difficult, as the compound must be administered intravenously four times a day for 14 days (delivering ~360 g/patient).

A number of screens of chemical libraries have been undertaken to identify therapeutic leads against the African trypanosome. These include a phenotypic screen that interrogated a library of FDA-approved drugs for anti-trypanosomal activity [16], as well as screens developed to identify inhibitors of essential parasite enzymes. A screen for UDP-Glc 4'-epimerase inhibitors using a small natural products library [17] and a screen of a commercial 134,500 compound library for trypanothione reductase inhibitors [18] are two examples of target-based screens used to identify lead compounds for therapeutic development. In the last few years, TbHK inhibitors have been explored as potential anti-parasitic compounds. Previous efforts to

identify TbHK inhibitors include the development of compounds based on models of the TbHK structure (predicted from homology studies of the yeast structure), and exploring the activity of HK inhibitors from other systems [4]. Here we have used a HTS of 220,223 compounds to identify new inhibitors of the parasite enzyme.

In our screens, we have identified several novel inhibitors of TbHK1. One compound, ebselen, was the most potent inhibitor from both the LOPAC validation screen and the HTS. Ebselen is a lipid-soluble seleno-organic compound that has been employed in clinical trials to assess its value in prevention of ischemic damage in brain hemorrhage and stroke [19,20]. Ebselen inhibits lipid peroxidation through a glutathione peroxidase-like action [21], but may act through other mechanisms as well. Notably, a single oral dose (100 mg/kg) of ebselen yields serum values of 4-5 μM [22] and brain levels of the drug reach 21% of plasma levels [23], suggesting that the compound (or its derivatives) may be useful for both early and late stage sleeping sickness therapy development.

Ebselen likely has polypharmacological effects on BSF parasites, as the compound is known to inhibit a number of enzymes in addition to TbHK1, including the trypanosome UDP-Glc 4'-epimerase [17]. Ebselen, unlike other cluster 1 compounds, has an IC_{50} that is significantly lower than the EC_{50}, suggesting its metabolism may be distinct from the sulfur-bearing compounds. Alternatively, a cellular "sink" could be interacting with ebselen, thereby lowering its effective concentration.

The remaining cluster 1 compounds have EC_{50}s notably lower than their TbHK1 IC_{50}s, suggesting possible actions on other cellular targets. Alternatively, differences between the two values could result from the concentration of the compound within the parasite (perhaps in the

glycosome) or metabolism of the inhibitor to a more potent form. These hypotheses would seem worthy of further investigation.

An ideal therapeutic drug for African sleeping sickness would target the parasite (while perhaps enhancing host immune responses) and work at concentrations low enough to limit the severity of side effects. In the search for potential drug targets, we have focused on the trypanosome TbHK1, a protein that the parasite requires to make ATP and have identified compounds that may serve as leads in for the development of therapeutics in the continuing fight against the African trypanosome.

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